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Folate (pteroylglutamate) uptake in human red blood cells, erythroid precursors and KB cells at high extracellular folate concentrations

Evidence against a role for specific folate-binding and transport proteins

Asok C. ANTONY,*‡ Madeleine A. KANE,† S. Ram KRISHNAN,* R. Stephen KINCADE* and Rama S. VERMA*

*Division of Hematology–Oncology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46223, and †Denver Veterans Administration Medical Center, Denver, CO 80220, U.S.A.

Membrane-associated folate (pteroylglutamate, PteGlu)-binding proteins (FBPs) play an important role as PteGlu-transport proteins in malignant and normal human cells. Since high extracellular folate (PteGlu) concentrations (EFC) profoundly influenced uptake and toxicity of the anti-PteGlu methotrexate in malignant KB cells, we studied human cells to determine additional mechanisms for PteGlu uptake when the EFC was varied. At low EFC (< 10 nM), the predominant mechanism for folate uptake in mature erythrocytes was through binding to externally oriented FBPs which were quantitatively insignificant (4–6 orders of magnitude lower) and of no apparent physiological relevance when compared with KB cells. However, the predominant mechanism of PteGlu accumulation at high EFC [10–250 nM] in intact erythrocytes and sealed right-side-out (RSO) ghosts was not FBP-mediated and non-specific. This conclusion was based on the findings that radiolabelled PteGlu uptake: (i) continued even in the presence of a 1000-fold excess of unlabelled PteGlu and was linear and not saturable up to 250 nM; (ii) was two-fold higher at pH 4.5 than 7.5; (iii) was less than 2-fold increased at 37 °C compared with 4 °C; and (iv) was unaffected after trypsin-mediated proteolysis of > 75% FBPs. The [^{3}H]PteGlu and ^{125}I-PteGlu (histamine derivative) accumulated intracellularly through the non-specific PteGlu-uptake mechanism was unaltered biochemically and in a soluble compartment. Raising the EFC 500-fold higher than controls during erythropoiesis *in vitro* resulted in reversal of the expected anti-(placental folate-receptor)-antiserum-induced megaloblastic changes in orthochromatic normoblasts derived from burst-forming unit-erythroid colonies. Furthermore, at EFC > 0.1 μM, KB-cell accumulation of [^{3}H]PteGlu was also predominantly through a mechanism that did not involve specific FBPs. Thus, at high EFC, a major component of PteGlu transport in human cells is not mediated through FBPs and is likely to be a passive diffusion process.

INTRODUCTION

We recently demonstrated that membrane-associated folate (pteroylglutamate, PteGlu)-binding proteins (FBPs) play an important functional role in PteGlu uptake in malignant human nasopharyngeal carcinoma KB cells in culture [1–3]. These proteins [2,4,5] have extensive biochemical similarity to solubilized particulate (P)-FBPs from human placenta [6] and milk [7]. In addition, we have also established that perturbation of similar immunoreactive FBPs on human erythroid progenitor cells with anti-[placental folate (PteGlu) receptor] (PFR) antiserum during erythropoiesis *in vitro* results in morphological and biochemical characteristics of PteGlu deficiency [8]. Thus high-affinity surface-membrane-associated FBPs are important PteGlu-transport proteins in cellular PteGlu uptake in both malignant and normal proliferating human cells.

Although methotrexate (MTX) binds the same PteGlu-transport protein as 5-methyltetrahydrofolate (5-CH_3H_4PteGlu) and PteGlu, high extracellular folate (PteGlu) concentrations (EFC) appeared to profoundly influence MTX uptake, intracellular MTX metabolism and MTX-induced cytotoxicity [9]. One explanation for these effects is the fact that PteGlu effectively competes with MTX for FBP-mediated binding and uptake, owing to its known higher affinity than MTX [5,10]. A second possibility is that high EFC down-regulated the content of KB-cell FBPs with the result that less MTX was accumulated intracellularly. Although we have recently demonstrated regulation of FBPs by varying the EFC [11], this process occurs over numerous cell passages and requires days to reach steady-state levels. The third possibility is that PteGlu enters KB cells independent of PteGlu-transport proteins. This has not been directly

Abbreviations used: FBP, folate (pteroylglutamate, PteGlu)-binding protein; S-FBP, soluble FBP; P-FBP, solubilized particulate FBP; ^{125}I-PteGlu (HD), ^{125}I-labelled PteGlu (histamine derivative); PFR, placental folate (PteGlu) receptor; EFC, extracellular folate (PteGlu) concentrations; PBS, phosphate-buffered saline [10 mM-potassium phosphate (pH 7.5)/150 mM-NaCl]; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; 5-CH_3H_4-, 5-methyltetrahydro-; 5-$HCOH_4$, 5-formyltetrahydro-; MTX, methotrexate (amethopterin); RBC, red blood cells (erythrocytes); BFU-E, burst-forming units-erythroid; RSO, sealed right-side-out; G3PD, glyceraldehyde 3-phosphate dehydrogenase.

‡ To whom correspondence and reprint requests should be sent, at: Clinical Building, Room 379, Indiana University School of Medicine, Indianapolis, IN 46223, U.S.A.

studied in KB or other human cells under conditions where the content of FBPs is not regulated and the ligand-binding site on FBPs has been specifically occupied by unlabelled ligand.

Despite the demonstration of functional FBPs on erythroid precursors [8], we have also recently shown that most immunoreactive FBPs (> 99%) in red-blood-cell (RBC) membranes purified from circulating (mature) RBC are non-functional in PteGlu binding; membranes from 1 ml of RBC bound < 10 fmol of an iodohistamine derivative of PteGlu [^{125}I-PteGlu (HD)] [12]. However, previous studies had shown that up to 3 pmol of authentic 5-CH_3H_4PteGlu was accumulated/ml of RBC [13,14]. It was therefore possible that our findings (with membranes) were not reflective of the content of FBPs on intact RBC if large quantities of functional FBPs were ('artifactually') released from membranes during (lysis of cells and) membrane preparation. Therefore the burden of proof rested on us to identify whether the earlier results [13,14] were attributable to FBP-mediated uptake mechanisms on intact mature RBC or to some other mechanism. Here, we report that, at high EFC, PteGlu can enter proliferating as well as non-proliferating cells by a process that is not solely dependent on FBP-mediated transport mechanisms and which is most compatible with passive diffusion.

MATERIALS AND METHODS

^{125}I-PteGlu (HD) (sp. radioactivity 2200 Ci/mmol) was from du Pont/NEN, Boston, MA, U.S.A. [3′,5′,7,9-^{3}H]folic acid ('[^{3}H]PteGlu') (sp. radioactivity 29 Ci/mmol) was from Amersham International. 5-CH_3[^{3}H]H_4PteGlu was prepared as described in [3]. PteGlu (99% pure), 5-formyltetrahydrofolate (5-$HCOH_4$PteGlu; 90% pure), MTX (90% pure), DL-5-CH_3H_4PteGlu (90% pure), Triton X-100, α-cellulose, Sigmacell Type 50, bovine pancreatic trypsin (Type XI), and chicken egg-white purified ovomucoid trypsin inhibitor were from Sigma Chemical Co., St. Louis, MO, U.S.A. Scintillation fluid (3a70) was from RPI, Mt. Prospect, IL, U.S.A.

PteGlu-uptake studies

(1) RBCs. All studies were performed on peripheral venous blood from normal volunteers with reticulocyte counts < 1%, and the haematocrit, RBC and leukocyte counts were measured by means of a Coulter counter (model M430; Coulter Electronics, Hialeah, FL, U.S.A.). A minor modification [6] of the method described by Beutler *et al.* [15] was used to purify leukocyte-free RBC. Routinely, RBC were sedimented by centrifugation at 1000 *g* for 10 min at 4 °C. The supernatant was aspirated and the pellet was resuspended in 15 vol. of ice-cold PBS. This centrifuge-wash cycle was repeated for a total of four times, and the final sample of packed RBCs had a haematocrit of 60%. A final volume of 2 ml contained 300 μmol of NaCl and 20 μmol of potassium phosphate, pH 7.5, and 1000 μl of RBC suspension, to which ^{125}I-PteGlu (HD) was added in increasing concentrations. Routine incubations, unless otherwise specified, were performed for 90 min at 37 °C (equilibrium binding) in a shaking waterbath, followed by cooling to 4 °C for 10 min. Unbound radioligand was completely removed by four centrifuge-wash cycles using 15 vol. of PBS per wash, as described above, and RBC were counted for radioactivity in a Beckman gamma 5500 γ-radiation counter at 78% efficiency. Additional samples which also contained a 1000-fold excess of unlabelled PteGlu were also routinely analysed for each level of radiolabelled PteGlu to assess non-specific PteGlu uptake into cells (RBC and KB cells) and ghosts. {Since ^{125}I-PteGlu (HD) and [^{3}H]PteGlu have high affinity and comparable specificity for the PteGlu-binding site in FBPs of RBC membranes and KB cells respectively, we used unlabelled PteGlu [rather than ^{125}I-PteGlu (HD)] to occupy available (common) ligand-binding sites on FBPs when assessing non-FBP-mediated (non-specific) uptake of ^{125}I-PteGlu (HD); the term 'non-specific PteGlu uptake' is used here to indicate that PteGlu uptake is not specifically FBP-mediated.} In pilot studies, sedimentation of cells through mineral oil after four centrifuge-wash cycles did not release additional radiolabel into the supernatant, indicating complete removal of extracellular radioligand.

Competitive inhibition of ^{125}I-PteGlu (HD) uptake by RBC with various unlabelled PteGlu species was performed with increasing concentrations of unlabelled PteGlu species (determined from absorption coefficients given by Blakley [16]) incubated with 0.025 nM-^{125}I-PteGlu (HD) and 1 ml of RBC in a final volume of 2 ml in PBS. Because of the relatively unstable nature of 5-CH_3H_4PteGlu [17], it was dissolved in PBS containing 100 mM-2-mercaptoethanol (2-ME) or 2 mM-dithiothreitol (DTT). Various concentrations of normal human plasma (filtered through a 0.22 μm-pore-size filter) was also tested for inhibitory activity.

To define the external orientation of FBPs on intact RBC, tubes containing 1 ml of RBC in PBS were incubated with 75 pM-^{125}I-PteGlu (HD), and after removal of unbound radioactivity, increasing concentrations of trypsin dissolved in 1 ml of PBS were added. Control experiments using trypsin which was first inactivated by preincubation for 30 min at 37 °C with a 2-fold excess of trypsin inhibitor were also carried out. After mixing, the samples were incubated at 37 °C for 1 h and after cooling to 4 °C, cell-associated radioactivity was determined as described above. To rule out the possibility that trypsin only displaced bound ^{125}I-PteGlu (HD) from intact RBC, increasing concentrations of trypsin (dissolved in 2 ml of PBS) were added to tubes containing 1 ml of RBC and the mixtures were incubated for 1 h at 37 °C. The samples were then centrifuged at 1000 *g* for 10 min at 4 °C, and the uppermost 1 ml of each supernatant was aspirated and boiled at 100 °C for 30 min in 2 mM-DTT. The remaining trypsin-treated RBC were subjected to three centrifuge-wash cycles, before radioligand-uptake studies were repeated. The inhibitory potential of the supernatants of trypsin-treated cells was tested by incubating the boiled supernatant at each level of trypsin with 1 ml of fresh washed untreated RBC and 75 pM-^{125}I-PteGlu (HD), after which cell-associated radioligand was determined.

(2) Sealed right-side-out (RSO) ghosts. RSO ghosts were prepared as described [18] by suspending lysed RBC membranes [12] at a 1:40 ratio with sealing buffer (150 mM-NaCl/10 μM-$MgSO_4$/5 mM-sodium phosphate, pH 8). After incubation for 1 h at 37 °C, RSO ghosts were sedimented at 30000 *g* for 7 min at 4 °C. After aspiration of the supernatant and two successive centrifuge-wash cycles with 40 vol. each of sealing buffer,

pH 5.5 and pH 8, the RSO ghosts were finally resuspended into a volume of sealing buffer (pH 8) such that each ml of RSO ghosts contained approx. 5 mg of protein. Glyceraldehyde 3-phosphate dehydrogenase (G3PD) accessibility assays [19,20] were performed on RSO ghosts to ensure they were inaccessible to G3PD ($\leq 10\%$ accessibility), indicating appropriate RSO sealing.

PteGlu uptake by RSO ghosts was carried out in 2 ml of sealing buffer. After incubation with various concentrations of ^{125}I-PteGlu (HD) or [^{3}H]PteGlu $\pm$ 1000-fold excess unlabelled PteGlu for 1 h at 37 °C, 5 ml of ice-cold sealing buffer was added and the sample centrifuged in Bio-vials (Beckman, Palo Alto, CA, U.S.A.) at 30000 *g* for 7 min at 4 °C. The supernatant was aspirated and the pellet was washed for four additional wash cycles with ice-cold sealing buffer and counted for radioactivity. Pellets containing [^{3}H]PteGlu were mixed with 10 ml of 3a70 scintillation fluid and counted for radioactivity in a Beckman LS 6800 liquid-scintillation counter at 50% efficiency.

To determine the optimum pH for radioligand uptake in RSO ghosts at high EFC, vials containing 1 ml of RSO ghosts in sealing buffer were centrifuged at 30000 *g* for 7 min at 4 °C. The pellets were resuspended with 40 nM-^{125}I-PteGlu (HD) or [^{3}H]PteGlu in a final volume of 2 ml containing 5 mM-sodium phosphate at various pH values ranging between 4.5 and 9, 150 mM-NaCl and 10 μM-$MgSO_4$. (The pH was adjusted in stock solutions by adding 0.1 M dibasic sodium phosphate to 0.1 M monobasic sodium phosphate; to achieve a pH of 4.5, 1 M-phosphoric acid was added to 0.1 M monobasic sodium phosphate.) After incubation for 1 h at 37 °C, the pellets were washed four times with 10 vol. of buffer per cycle, using the same pH buffer with which the incubation with radioligand was carried out, and then counted for radioactivity.

To analyse whether the internalized radioactivity into RSO ghosts was authentic radiolabelled PteGlu, [^{3}H]PteGlu uptake into RSO ghosts (at increasing EFC $\pm$ 1000-fold excess unlabelled PteGlu) was first studied as described above. After the final wash, the ghost pellets were permeabilized by incubation for 10 min at 4 °C with 1 ml of 0.01 M-KPO_4, pH 7.5, containing 0.1% Triton X-100 (confirmed by 100% accessibility to G3PD). The permeabilized ghosts were sedimented at 30000 *g* for 30 min at 4 °C, 1 ml of each supernatant was aspirated and individually filtered through 0.2 μm-pore-size Millex-GV filters. The filtrates (900 μl) were then mixed with 200 ng of purified human PFR [6], which had a capacity to bind 5 pmol of PteGlu. After incubation for 30 min at 37 °C, dextran-coated charcoal (40 mg) was added, and the mixtures incubated at 4 °C for 10 min. The samples were centrifuged at 30000 *g* for 30 min at 4 °C, and 1 ml of each supernatant (containing [^{3}H]PteGlu bound to PFR) was counted for radioactivity. A similar experiment was also performed using ^{125}I-PteGlu (HD) substituted for [^{3}H]PteGlu.

(3) Studies in cultured human KB cells. The culture conditions, harvesting and folate uptake studies on KB cells were carried out exactly as described [3] except that the EFC ranged from 1 nM to 10 μM.

(4) Studies during erythropoiesis *in vitro*. Cultures of burst-forming unit-erythroid (BFU-E) colonies in the presence of rabbit preimmune and anti-PFR antiserum were carried out exactly as described in [8]. To achieve an approx. 500-fold excess EFC concentration compared with control plates containing preimmune and anti-PFR antiserum, unlabelled PteGlu (2.8 mM in PBS) was added to additional samples prepared in the presence of preimmune and anti-PFR antiserum. At day 14, BFU-E-derived colonies were plucked, transferred to slides, and directly stained (without prior fixing), and quantitative and qualitative morphological studies were carried out as described in [8]. Avoiding the cell-fixation step resulted in less shrinking of the cells.

Purification and analysis of radiolabelled PteGlu species

[^{3}H]PteGlu, 5-CH_3[^{3}H]H_4PteGlu and ^{125}I-PteGlu (HD) were purified > 99% by h.p.l.c. as previously described [3,5,12].

Calculations

Each assay was routinely performed in triplicate, and the results are expressed as the mean when there was no

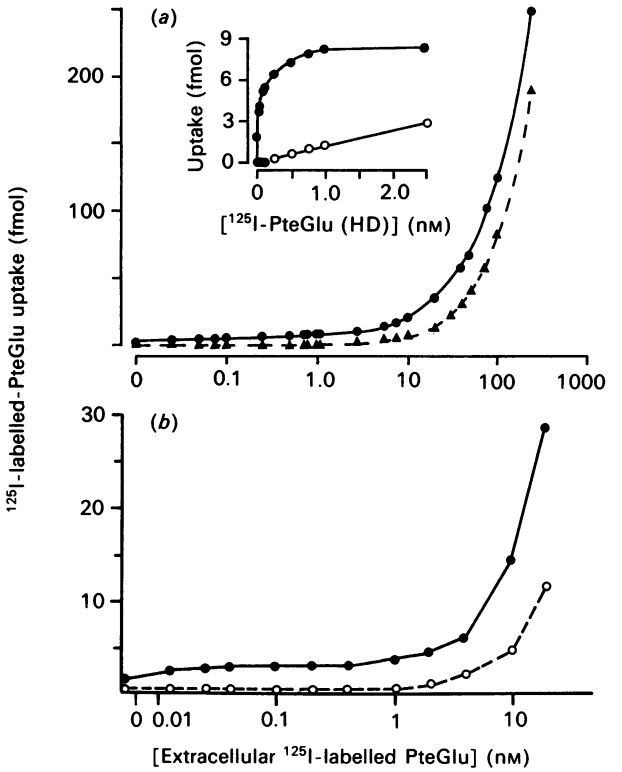

Fig. 1. Total and non-specific PteGlu uptake by intact RBCs and RBC ghosts at various extracellular concentrations

(*a*) RBCs (1 ml) were incubated with increasing concentrations of ^{125}I-PteGlu (HD) in the absence (●) or presence (▲) of a 1000-fold excess of unlabelled PteGlu for 1 h at 37 °C. They were subsequently washed four times with 14 vol. of PBS and counted for radioactivity. Inset: Specific (●) and non-specific (○) radioligand uptake at ^{125}I-PteGlu (HD) concentrations < 2.5 nM. (*b*) RSO ghosts, 1.5 ml, were incubated in sealing buffer with increasing concentrations of ^{125}I-PteGlu (HD) in the absence (●) or presence (○) of a 1000-fold excess of unlabelled PteGlu in a final volume of 2 ml of buffer containing 10 μmol of sodium phosphate, pH 8, 300 μmol of NaCl and 20 nmol of $MgSO_4$. After incubation for 1 h at 37 °C, the ghosts were washed with 10 vol. of buffer four times and counted for associated radioactivity. The abscissa is a logarithmic scale.

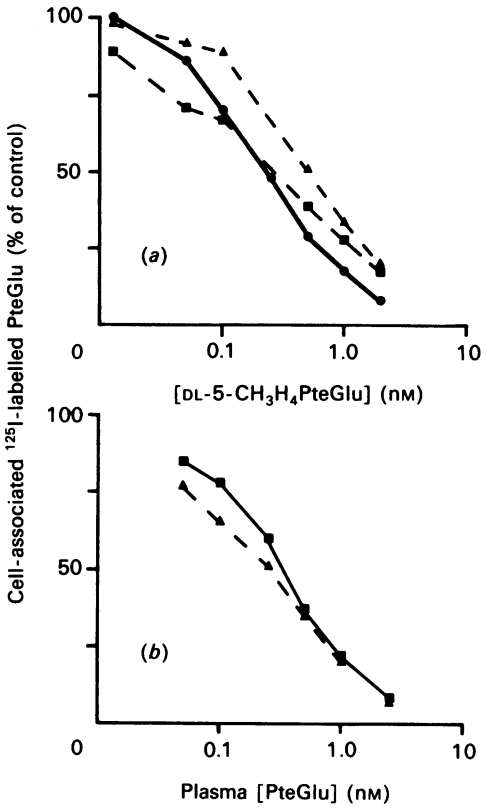

Fig. 2. Competitive inhibition of ^{125}I-PteGlu (HD) binding to 1 ml of RBCs by DL-5-CH_3H_4PteGlu (*a*) and human plasma (*b*)

Radioligand-uptake studies using 0.025 nM-^{125}I-PteGlu (HD) were performed in the absence (●) or presence of 100 mM-2-ME (■) or 2 mM-DTT (▲). The maximal uptake (100%) was determined in the absence of unlabelled PteGlu, but in the same concentration of 2-ME or DTT as the test samples.

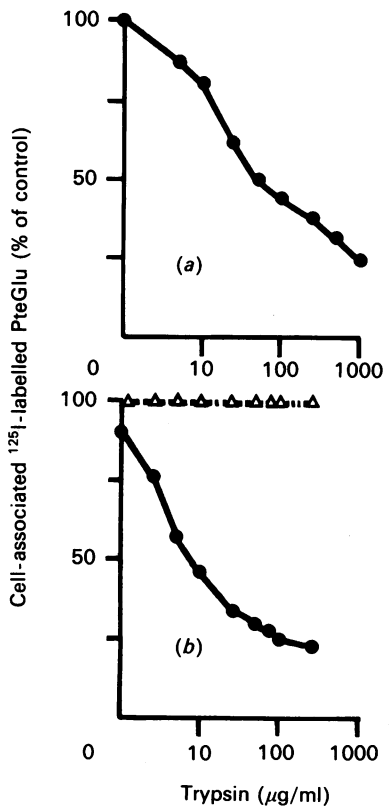

Fig. 3. Effect of trypsin on specific uptake of ^{125}I-PteGlu (HD) by RBCs

Cells containing bound ^{125}I-PteGlu (HD) were incubated with increasing concentrations of trypsin for 1 h at 37 °C (*a*). After treatment with increasing concentrations of trypsin for 1 h at 37 °C, erythrocytes were washed to remove excess trypsin and digested externally oriented membrane proteins, followed by incubation with radioligand (*b*). The data (●) are expressed as a percentage of maximum uptake obtained with control samples using inactivated trypsin (not shown). Each supernatant (after treatment of cells with different concentrations of trypsin) was also tested for its inhibitory effect on subsequent radioligand uptake by another batch of untreated cells (△). The 100% control value (no inhibition), obtained by using the supernatant from cells incubated with inactivated trypsin, was 5.75 fmol/ml of erythrocytes. Non-specific binding of ^{125}I-PteGlu (HD) was less than 10% of total ^{125}I-PteGlu (HD) binding (not shown).

greater than 5% deviation from the mean in each sample. Non-specific PteGlu uptake was defined as radiolabelled-PteGlu uptake in the presence of a 1000-fold excess of unlabelled PteGlu, and total radiolabelled PteGlu uptake was the result obtained in the absence of competing unlabelled PteGlu [see the note in face brackets under '(1) RBCs' above]. Specific FBP-mediated PteGlu uptake was defined as the difference between total PteGlu uptake and non-specific PteGlu uptake. The IC_{50}, defined as the concentration of folate analogue at which RBC ^{125}I-PteGlu (HD) uptake was decreased to 50%, was determined directly from competitive-inhibition curves.

RESULTS

Specific PteGlu uptake by RBC and RSO ghosts

Fig. 1 shows the effect of extracellular radioligand concentration on ^{125}I-PteGlu (HD) uptake at 37 °C by intact RBC and RSO ghosts. At ^{125}I-PteGlu (HD) concentrations below 1 nM the predominant uptake mechanism was a specific, saturable, high-affinity mechanism with a K_d of 33 pM. Fig. 2 shows the inhibitory effect of commercially available DL-5-CH_3H_4PteGlu and the folate from human plasma (predominantly L-5-CH_3H_4PteGlu) on uptake of ^{125}I-PteGlu (HD) by intact RBC. The concentrations of DL-5-CH_3H_4PteGlu and plasma PteGlu which inhibited net radioligand uptake by 50% (IC_{50}) were 0.2 nM and 0.4 nM respectively. Whereas equimolar concentrations of unlabelled PteGlu added together with 0.025 nM-^{125}I-PteGlu (HD) resulted in 50% inhibition of net radioligand uptake by RBC, the IC_{50} values for 5-$HCOH_4$PteGlu and MTX were much higher, being 6.5 nM and 10 nM respectively (results not shown). These data, indicating a higher affinity of RBC FBPs for oxidized than for reduced PteGlu species, are comparable with those reported for FBPs from human milk [7], placenta [6], KB cells [1–3,5,10] and RBC membranes [12]. Fig. 3(*a*) shows the dose-dependent effect of trypsin treatment of intact RBC on the subsequent release of bound ^{125}I-PteGlu (HD) from cells. The dose-dependent decrease in the capacity of RBC to

Table 1. Release of specifically bound ^{125}I-PteGlu (HD) from RBC membranes and RSO RBC ghosts after various pH washes

A 1.25 ml portion of membranes or ghosts was incubated with 0.025 nM-^{125}I-PteGlu (HD) in the absence or presence of a 1000-fold excess of unlabelled PteGlu at pH 7.5 for 1 h at 37 °C. After four centrifuge-wash cycles with pH 7.5 buffers, they were washed with either pH 7.5 or 5.5 buffer for two final wash cycles before counting for radioactivity.

pH of final two washes*	Radioligand binding by membranes (fmol)	Radioligand uptake by ghosts (fmol)
7.5	5.1	6.7
5.5	0.5 (10%)†	2.3 (34%)†

* Membranes and ghosts were washed with lysis and sealing buffers respectively.

† Values in parentheses indicate the percentage of maximal radioligand (taken up at pH 7.5) which was retained after washing with pH 5.5 buffer.

bind ^{125}I-PteGlu (HD) after trypsin treatment is shown in Fig. 3(*b*). That the decrease in ^{125}I-PteGlu (HD) binding was not due to release of PteGlu from intracellular stores due to trypsin-induced haemolysis is demonstrated by the inability of the (boiled) supernatant of trypsin-treated RBC to inhibit ^{125}I-PteGlu (HD) binding to untreated RBC (Fig. 3). These data suggest that, at low EFC, ^{125}I-PteGlu (HD) binding was to externally oriented trypsin-sensitive FBPs. These findings are pertinent to the interpretation of previous studies [14], which demonstrated PteGlu uptake in trypsin-treated RBCs (discussed below).

By using RSO ghosts, experiments were subsequently carried out to differentiate ligand binding to the plasma-membrane surface from internalized ligand. As shown in Table 1, more than 90% of ^{125}I-PteGlu (HD) specifically bound to membrane preparations was released after exposure to acidic buffer. However, only 66% of ^{125}I-PteGlu (HD) associated with RSO ghosts was released after exposure to iso-osmotic pH 5.5 buffer. G3PD accessibility in ghosts treated with iso-osmotic pH 5.5 buffer was $\leqslant 10\%$ of that activity in a comparable amount of RBC membranes, indicating maintenance of the integrity of sealing in RSO ghosts [20]. Thus specific acid-sensitive binding sites for ^{125}I-PteGlu (HD) were located externally on RSO ghosts, and 34% of accumulated radioactivity was acid-insensitive.

As shown in Table 2, although the total amount of ^{125}I-PteGlu (HD) specifically associated with RSO ghosts decreased by 21% when ghosts were washed seven times at pH 7.5 (expt. C) compared with four washes (expt. A), this was entirely due to a decrease in the acid-insensitive portion of the ghost-associated radioactivity (expt. B versus expt. D). Although RSO ghosts remained intact, as evidenced by no change in G3PD accessibility, the amount of acid-sensitive ghost-associated radioactivity remained unchanged after four or seven washes at pH 7.5 (expts. A minus B = 9970 c.p.m., and expts. C minus D = 9932 c.p.m.). Thus efflux of radioactivity from RSO ghosts appeared to be responsible for the decrease in acid-insensitive ^{125}I-PteGlu (HD) after seven washes as compared with four washes. Subsequent treatment of RSO ghosts with buffer containing 0.1% (v/v) Triton X-100 resulted in complete permeabilization of ghosts. Preliminary studies (not shown) revealed that ghosts permeabilized with 0.1% Triton X-100 for 10 min continued to bind ^{125}I-PteGlu (HD) with an affinity comparable with that of RBC membranes [12]; thus Triton X-100 did not alter the K_d of RBC FBP–radioligand interaction. As shown in Table 2, expt. E, the amount of ^{125}I-PteGlu (HD) bound to permeabilized ghosts was the same as the amount bound to the RSO-ghost surface in expts. A and C. Furthermore, after the release of surface-bound radioactivity at pH 5.5, followed by permeabilization of the ghosts (expt. F), less than 10% of the original ghost-associated radioactivity (expt. C) remained bound. Thus permeabilization of RBC ghosts by 0.1% Triton X-100 permitted efflux of most of the acid-insensitive radioactivity, since it was not significantly associated with internal membrane-associated binding

Table 2. Total, surface-bound and internalized ^{125}I-PteGlu (HD) in RBC ghosts assessed by washing ghosts with sealing buffer at various pH values, before and after Triton X-100-mediated permeabilization

	No. of washes at the indicated pH*							
Expt.	7.5	5.5	7.5† (*a*)	5.5† (*b*)	7.5† (*c*)	7.5	Total no. of washes	^{125}I-PteGlu (HD) uptake (c.p.m.)
A	4	—	—	—	—	—	4	16117‡
B	2	2	—	—	—	—	4	6147‡
C	4	—	—	—	1	2	7	12673§
D	2	2	—	1	—	2	7	2741§
E	4	—	1	—	—	2	7	10055§
F	2	2	1	—	—	2	7	830§

* A 0.75 ml portion of ghosts was incubated with 25 fmol of ^{125}I-PteGlu (HD) for 1 h at 37 °C in a final volume of 1 ml of sealing buffer, pH 7.5; they were then washed with sealing buffers at indicated pH and counted for radioactivity; the washes were performed sequentially from left to right in various buffers.

† Ghosts were incubated for 10 min at 4 °C in 10 vol. of indicated-pH sealing buffer in the presence (*a*) or absence (*b* and *c*) of 0.1% Triton X-100, after which wash cycles were continued as shown.

‡ Mean for six samples with less than 5% deviation from the mean.

§ Mean for three samples with less than 5% deviation from the mean.

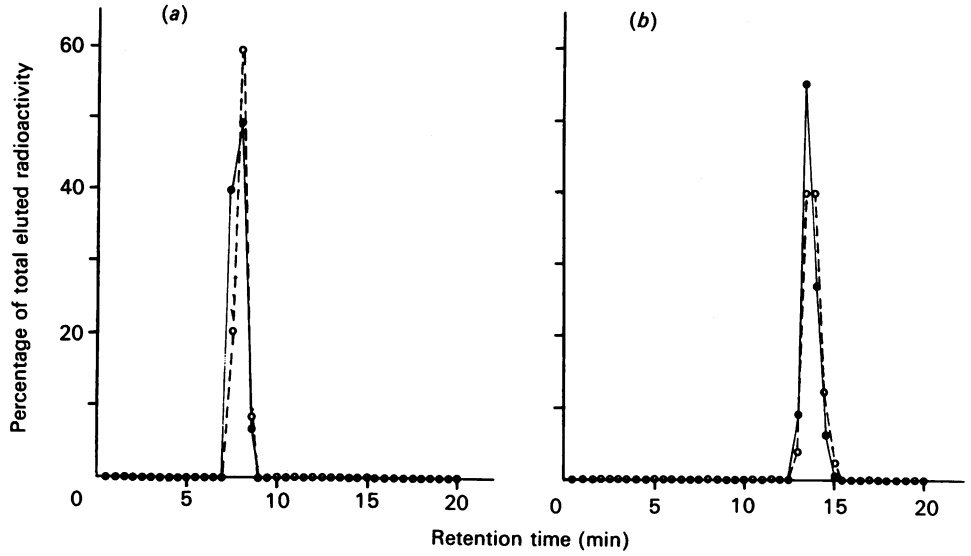

Fig. 4. H.p.l.c. analysis of (*a*) ^{125}I-PteGlu (HD) and (*b*) [^{3}H]PteGlu

An aliquot of radiolabelled PteGlu species (●; 0.5 pmol) was purified as described in the Materials and methods section and applied over C_{18} μBondapak stainless-steel (*a*) or Radial-pak (*b*) columns at a flow rate of 1 ml/min in equilibration and elution buffer consisting of 5 mM-Pic A, 10 mM-KH_2PO_4/H_3PO_4, pH 5.5 (Buffer A) and acetonitrile (Buffer B) (21:4, v/v), respectively. After permeabilization of RBC ghosts previously incubated with 80 nM-radioligand, an aliquot of released radioactivity was analysed (○).

sites. Moreover, with 0.1% Triton X-100, insignificant membrane-associated radioligand was released out of RBC membranes. These studies established the maximal contribution of FBPs to the total amount of PteGlu accumulated by RSO ghosts at low EFC. They also showed that any additional PteGlu uptake by RSO ghosts at higher EFC would be due to radioligand accumulated intracellularly by non-FBP-mediated mechanisms.

Non-specific PteGlu uptake by RBC and RSO ghosts

As shown in Fig. 1, when intact RBC and RSO ghosts were incubated with ^{125}I-PteGlu (HD) at concentrations greater than 10 nM, 50% or more of the cell- or RSO-ghost-associated radioactivity was taken up non-specifically, i.e., not blocked in the presence of a 1000-fold excess of unlabelled PteGlu. As the ^{125}I-PteGlu (HD) concentration was increased, the proportion of radioligand taken up by the non-specific mechanism (in RBC and RSO ghosts) rose very rapidly, whereas the proportion of total ^{125}I-PteGlu (HD) taken up which was contributed to by specific binding to FBPs was not changed. When duplicate samples of ghosts incubated with high concentrations of ^{125}I-PteGlu (HD) were washed with two centrifuge-wash cycles of pH 5.5 buffer, the net released radioactivity was similar to that predicted from ligand bound to the external surface of ghosts (not shown), and the resulting profile was identical with the curve generated in Fig. 1(*b*) with a 1000-fold excess of unlabelled PteGlu. Thus these data suggested that the ghost-associated radioactivity was not accessible to acid-stripping and was probably internalized (see below). Non-specific radioligand uptake was not saturable, as emphasized by the linear increase in RBC- and RSO-ghost-associated radioligand with increasing extracellular ^{125}I-PteGlu (HD) concentration, even in replicates containing 1000-fold excess non-radioactive PteGlu; in the latter samples, the actual total amount of ligand (labelled and unlabelled PteGlu) taken up would be almost 1000-fold more than in RBC or RSO ghosts incubated with radioligand alone. Similar results on non-specific PteGlu uptake were observed when highly purified [^{3}H]PteGlu (Figs. 4 and 5) and 5-CH_3[^{3}H]H_4PteGlu (not shown), which are not chemically modified [as is the histamine derivative of folate, ^{125}I-PteGlu (HD)], were used. However, because of the much lower specific activity of [^{3}H]PteGlu and 5-CH_3[^{3}H]H_4PteGlu, the specific uptake of these radioligands contributed by binding to FBPs on RBC membranes could not be easily quantified. At an EFC greater than 10 nM-^{125}I-PteGlu (HD), RBC radioligand uptake in the presence of 12.5 nM plasma PteGlu was similar to the non-specific PteGlu-uptake curve (Fig. 1*a*) up to concentrations of 250 nM (not shown). After trypsin treatment, more than 75% of the FBPs on the surface of intact RBC lost their capacity to bind ^{125}I-PteGlu (HD) (Fig. 3); however, the amount of non-specific PteGlu uptake was also unaffected by trypsin treatment (not shown) and was similar to the profile shown in Fig. 1(*a*), where there was a 1000-fold excess of unlabelled PteGlu. These data suggest that it is highly likely that intact RBC do not significantly accumulate radiolabelled PteGlu in the nanomolar range by a specific FBP-mediated uptake mechanism; in addition, the non-specific PteGlu-uptake process may be operative in RBCs *in vivo*, since it occurred in the presence of normal human plasma.

At EFC of 80 nM-[^{3}H]PteGlu, there was RSO-ghost-associated uptake of 3.25 pmol and 3 pmol of radioligand in the absence and presence of a 1000-fold excess of unlabelled PteGlu respectively. Subsequent permeabilization of RSO ghosts previously incubated with 80 nM-[^{3}H]PteGlu resulted in release of 99% of the ghost-associated radioactivity, indicating that only a fraction remained bound to membrane-associated binding sites. The internalized radioactivity was identical with the

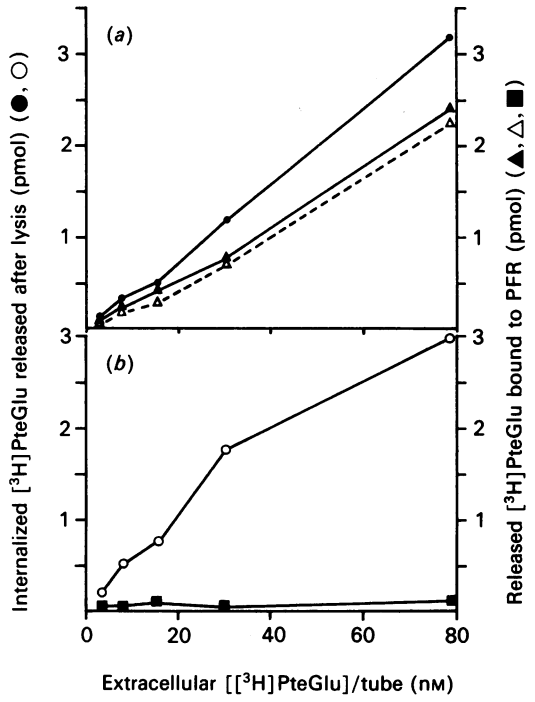

Fig. 5. Binding of radioactivity internalized to purified human PFR

After saturability studies to assess total and non-specific [^{3}H]PteGlu uptake in the absence (*a*, ●) and presence (*b*, ○) of a 1000-fold excess of unlabelled PteGlu respectively, 0.75 ml of RSO ghosts containing internalized radioactivity were permeabilized with 1 ml of 0.01 M-KPO_4, pH 7.5, containing 0.1% Triton X-100, and membranes were separated by centrifugation and filtration. Each filtered supernatant containing released radioactivity (900 μl) was then incubated with 200 ng of purified PFR for 30 min at 37 °C. Unbound radioactivity, adsorbed by dextran-coated charcoal, was separated by centrifugation, and 1 ml supernatants containing PFR-bound [^{3}H]PteGlu were counted for radioactivity. The supernatant from permeabilized ghosts in which a 1000-fold excess of unlabelled PteGlu was added (during initial incubation with radioligand) assessed non-specific binding of [^{3}H]PteGlu to PFR (■), whereas those incubated with [^{3}H]PteGlu alone assessed the total (specific plus non-specific) radioactivity bound to PFR (▲). Specific [^{3}H]PteGlu binding to PFR at each level of internalized radioligand (△) was obtained from the difference between total and non-specific radioligand binding to PFR.

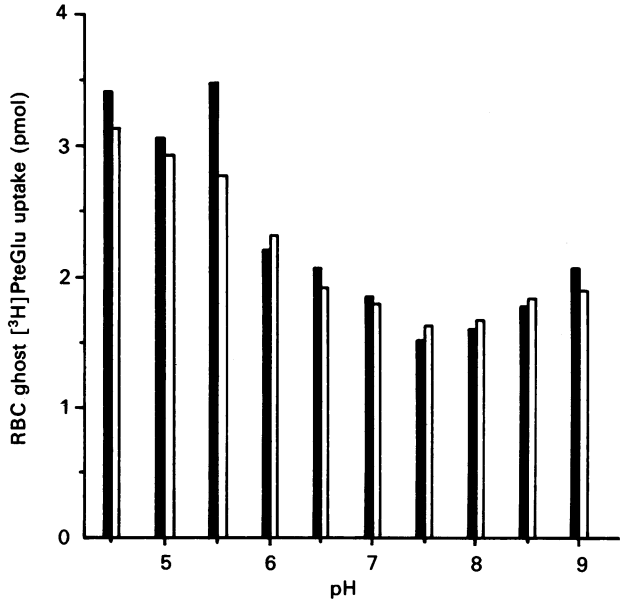

Fig. 6. pH-dependence of the non-specific PteGlu-uptake mechanism in RBC ghosts

Ghosts (1 ml) were incubated with 40 nM-[^{3}H]PteGlu in buffers prepared at various pH values as described in the Materials and methods section, in the absence (open bars) and presence (solid bars) of a 1000-fold excess of unlabelled PteGlu for 1 h at 37 °C, and ghost-associated radioactivity was determined. G3PD accessibility was < 10% at the end of these experiments.

authentic radioligand shown by its h.p.l.c. profile in Fig. 4. Furthermore, more than 90% of the [^{3}H]PteGlu released from permeabilized ghosts was functionally active and specifically bound human PFRs (Fig. 5).

The effects of buffer, temperature and pH on non-specific [^{3}H]PteGlu uptake in RSO ghosts were subsequently determined. It has been shown for RBC membranes [12] that dissociation of ^{125}I-PteGlu (HD) from FBP is almost complete (> 90%) at pH 5.5 or less. On the basis of G3PD accessibility studies we determined that exposure of RSO ghosts to iso-osmotic pH 5.5 buffer for 10 min at 4 °C did not lead to loss of integrity of sealing, which would be manifested by a rise in G3PD accessibility. Fig. 6 shows that non-specific [^{3}H]PteGlu uptake in RSO ghosts at pH 4.5 was 2-fold greater than at pH 7.5. Since radioligand binding to FBPs is minimal at this pH (Table 1 and [12]), experiments on non-specific radioligand uptake carried out at pH 5.5 would not involve FBP–PteGlu interaction. A 1.5-fold increase in non-specific [^{3}H]PteGlu uptake at 28 °C compared with that at 4 °C was observed, with no further increase at 37 °C (Fig. 7*a*). Fig. 7(*b*) shows that the rate of non-specific [^{3}H]PteGlu uptake increased with temperature, but Fig. 7(*c*) shows that the rate of efflux was unaffected by temperature. Whereas 50% of the internalized radioactivity was lost by 90 min, approx. 30% was still retained after 24 h, indicating an initial rapid efflux followed by a much lower rate of efflux.

Non-specific PteGlu uptake during erythropoiesis *in vitro*

We have shown that, in the presence of anti-PFR antiserum, but not in preimmune serum, the BFU-E-derived orthochromatic normoblasts contained 70% less intracellular PteGlu and had correlated morphological features consistent with nuclear-cytoplasmic asynchrony, which is the hallmark of megaloblastic erythropoiesis [8]. In the present study, we restricted our analysis to quantitative changes of (megaloblastic) morphology of these cells developed in the presence of anti-PFR antiserum in a mixture containing standard quantities of PteGlu (5.46 μM) or an approx. 500-fold excess of PteGlu (2.8 mM). As shown in Table 3, when 2.8 mM unlabelled PteGlu was included in the incubation mixture during the entire 14 days of culture, there was complete abrogation of the expected functional effects of anti-PFR antiserum (manifested by megaloblastic changes). The cells under these conditions had similar features to cells

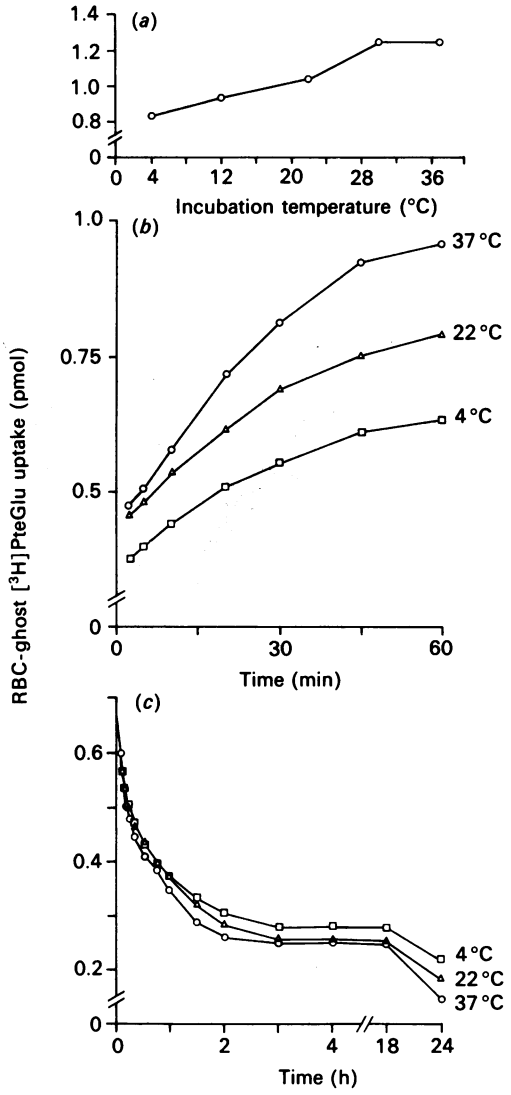

Fig. 7. Temperature-dependence of the non-specific PteGlu uptake mechanism in RBC ghosts

(*a*) Ghosts (1 ml) were incubated with 20 nM-[^{3}H]PteGlu at the temperatures indicated for 1 h and assessed for associated radioactivity. (*b*) Ghosts (0.65 ml) were incubated with 55 nM-[^{3}H]PteGlu at 4, 22 or 37 °C and at various times were washed and assessed for radioactivity. (*c*) Ghosts (0.5 ml) were incubated with 40 nM-[^{3}H]PteGlu for 15 min at 37 °C and, at various times, assessed for retained radioactivity after two 10 vol. washes with ice-cold sealing buffer, pH 8.

developed in the presence of preimmune serum. These results strongly suggest that the non-specific PteGlu-uptake process by-passed the anti-PFR antiserum-induced block in PteGlu uptake by BFU-E and their derived colonies during erythropoiesis *in vitro*

Non-specific PteGlu uptake by cultured human KB cells

The FBPs that are involved in PteGlu transport in human KB cells have been well characterized and purified [1–3,5]. In pilot experiments there was no change in the cellular content of immunoreactive FBPs [24] when KB cells in suspension were incubated in 0.1 μM- and 10 μM-PteGlu over 1 h at 37 °C (results not shown). In addition, when membrane preparations were made from these cells [3] and endogenous PteGlu was dissociated by washing membranes with pH 3 buffer [10], Scatchard analysis of [^{3}H]PteGlu binding to membrane-associated FBPs was unchanged (results not shown). Thus at low (0.1 μM) and high (10 μM) EFC, the immunoreactive as well as functional PteGlu-binding capacity of KB-cell FBPs was unchanged over short incubation times i.e. there was neither regulation or changes in their binding affinity for [^{3}H]PteGlu. At [^{3}H]PteGlu concentrations greater than 0.1 μM, most of the additional [^{3}H]PteGlu uptake (over that which was bound to high-affinity FBPs) was non-specific, since it was not blocked by the presence of 1000-fold excess unlabelled PteGlu (Fig. 8). Specific FBP-mediated [^{3}H]PteGlu uptake, on the other hand, was completely quenched by 1000-fold excess of unlabelled PteGlu at extracellular [^{3}H]PteGlu concentrations of less than 0.1 μM. Thus, at high PteGlu accumulation by KB cells occurs predominantly by a non-specific mechanism that is independent of a specific PteGlu-transport protein-mediated process. Furthermore, as in the case with intact RBC and ghosts (Fig. 1), a relatively constant percentage of extracellular radioactivity was accumulated by KB cells at each EFC into the micromolar range (Fig. 8).

DISCUSSION

In the present study we have shown that FBPs on intact RBC: (i) are quantitatively as well as qualitatively comparable with those reported previously for RBC membranes [12]; (ii) are externally oriented on intact RBC on the basis of trypsin-sensitivity and ligand binding dissociation at acidic pH; (iii) account for only a small (femtomolar) quantity of PteGlu accumulation in RBC and ghosts at low EFC, and are therefore not likely to be of physiological importance in mature human RBC. There is, however, a major controversy on the quantitative uptake of PteGlu species by mature RBC. By using low EFC, Corcino *et al.* [28] concluded that mature RBC did not take up significant amounts of PteGlu species. However, the significantly higher 5-CH_3H_4PteGlu [13,14] and MTX [29] uptake demonstrated in RBC at high EFC has been presumed to occur through a carrier-mediated substrate-specific temperature-dependent process that is influenced by counter-transport; whereas uptake of 5-$HCOH_4$PteGlu, 5-CH_3H_4PteGlu and MTX was similar, uptake of PteGlu was very poor. These and other similar data [30–33] have been presented as supportive evidence favouring independent transport systems for reduced PteGlu species and PteGlu. Huennekens *et al.* [34] re-evaluated studies with L-1210 cells and concluded that the earlier reports favouring a 'two transport-system hypothesis' were erroneously caused by a radiolabelled impurity in the PteGlu preparations used; on the basis of kinetic studies, the consensus [34] now favours a single PteGlu-transport system in L-1210 cells. Independent studies from our [1–5] and other [10] laboratories with human KB cells, erythroid cells [8,12] and monkey kidney cells [35] also concur with the view that membrane-bound FBPs on these cells bind PteGlu and 5-CH_3H_4PteGlu with high affinity, and intracellular transport of both of these PteGlu species are comparable and mediated by these FBPs. However, since Branda *et al.* [13] showed that the internalized radioactivity in mature RBC corresponded to authentic PteGlu, there is little doubt that nanomolar

Table 3. Effect of increasing the extracellular PteGlu concentration on anti-PFR-antiserum-induced megaloblastic changes during erythropoiesis *in vitro*

Each value represents the mean ± S.D. of each parameter studied of 200 orthochromatic normoblasts composing erythropoietic bursts in each treatment group from a single normal volunteer's bone-marrow aspirate. Morphometric studies were carried out by a 'blinded' observer.

Addition to culture	Cell size (μm)	Normoblasts having > one nucleus (%)	Mean no. of nuclei/cell
1. Rabbit preimmune serum	21.05 ± 5.65	4	1.08 ± 0.15
2. Rabbit preimmune serum plus 2.8 mM-PteGlu	21.69 ± 5.27	6.5	1.11 ± 0.37
3. Rabbit anti-PFR antiserum	27.44 ± 6.05†	16	1.31 ± 0.84**
4. Rabbit anti-PFR antiserum plus 2.8 mM-PteGlu	21.39 ± 4.73	4.5	1.06 ± 0.21

† $P < 0.001$ compared with all other experimental groups.
** $P < 0.05$ compared with all other experimental groups.

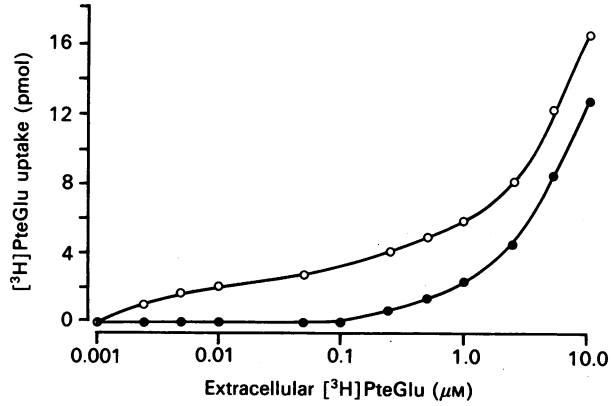

Fig. 8. Total and non-specific PteGlu uptake by cultured human KB cells

KB cells (1×10^6) were incubated with increasing concentrations of [^{3}H]PteGlu in the absence (○) or presence (●) of a 1000-fold excess of unlabelled PteGlu for 1 h at 37 °C and assessed for cell-associated radioactivity as described in [3]. The abscissa is a logarithmic scale.

concentrations of 5-CH_3H_4PteGlu were transported into these cells. Subsequent studies [14] revealed that trypsin treatment of RBC did not affect 5-CH_3H_4PteGlu uptake at an EFC of 2 μM. However, at the concentrations of trypsin used [14], our data (Fig. 3) would predict a loss of > 74% RBC FBPs, suggesting that 5-CH_3H_4PteGlu uptake (as previously reported [13,14]) was not mediated by these FBPs. Moreover, on the basis of the efficiency of the RBC PteGlu-transport system for various PteGlu species reported previously [13,14,29–33], one would predict the existence of a carrier FBP that has correlated higher affinity for 5-CH_3H_4PteGlu, 5-$HCOH_4$PteGlu and MTX than PteGlu. Interestingly, we find in contrast that RBC FBPs bind PteGlu and 5-CH_3H_4PteGlu with much higher affinity than 5-$HCOH_4$PteGlu and MTX (Fig. 2; [12]). Furthermore, we have been unable to demonstrate specific 5-CH_3[^{3}H]H_4PteGlu binding to RBC membranes using high-specific-radioactivity (50 Ci/mmol) radioligand due to the paucity of RBC FBPs. On the basis of our data, the quantity of specific ^{125}I-PteGlu (HD) binding (< 10 fmol/ml of RBCs), could not possibly account for the 1000-fold higher RBC accumulation of 5-CH_3H_4PteGlu previously reported (up to 3.5 pmol/ml of RBCs). It must be pointed out that the EFC (of 5-CH_3H_4PteGlu) used previously [13,14] were 100-fold higher than the physiological serum PteGlu level. Of major significance in the interpretation of previous data [13,14,32,33], is the fact that studies to determine the specificity of 5-CH_3H_4PteGlu uptake using an excess of unlabelled 5-CH_3H_4PteGlu with radiolabelled 5-CH_3H_4PteGlu (to quantitatively measure non-specific 5-CH_3H_4PteGlu uptake) were not reported. Therefore the data reported by Branda *et al.* [13] do not give any indication of whether a major component of total 5-CH_3H_4PteGlu uptake was contributed by non-specific uptake mechanisms. We, on the other hand, observed that, although specific ^{125}I-PteGlu (HD) uptake in mature RBC (as mediated by FBPs) is minimal, there was a major and significant component of non-specific uptake when the EFC was increased > 10 nM (Fig. 1). Under these conditions, radioligand binding to membrane-associated FBPs remained unchanged, whereas additional PteGlu uptake was predominantly accounted for by intracellular PteGlu. The non-specific PteGlu uptake, which was due to the accumulation of authentic, biologically unaltered and functionally active PteGlu, had rapid influx and efflux kinetics, was maximal at pH 4.5, and was slightly affected by temperature. The discovery that the percentage of intracellular PteGlu accumulated by this process was directly in proportion to the EFC {3 ± 1% of the EFC for [^{3}H]PteGlu, 0.1% of the EFC for ^{125}I-PteGlu (HD) and 0.3% of the EFC for [^{3}H]PteGlu} is consistent with non-mediated passive diffusion, since it was linear and non-saturable in sealed ghosts, intact RBC and KB cells.

Despite the anti-PFR-antiserum-mediated block in specific PteGlu uptake during erythropoiesis *in vitro*, inclusion of a 500-fold excess of unlabelled PteGlu resulted in complete abrogation of the expected megaloblastic changes. Thus despite (i) occupation of the ligand-binding site on PteGlu receptors on erythroid precursors by excess unlabelled PteGlu, and (ii) perturbation of these PteGlu receptors by anti-PFR antiserum, the fact that the cells accumulated adequate quantities of PteGlu (to avoid becoming megaloblastic) indicates the existence of another mechanism for PteGlu

uptake that is not specifically FBP-mediated; this is likely to be similar to the non-specific PteGlu-uptake process described with mature RBC (Fig. 1). The EFC required to effect non-specific PteGlu uptake during erythropoiesis *in vitro* was significantly higher than that which would have been predicted from Fig. 1. However, it should be appreciated that it may not be appropriate to compare EFC in semi-solid (methylcellulose) media and aqueous buffers. The concentration of erythropoietin routinely used [8,36] in stimulating BFU-E (10^5 low-density mononuclear cells/plate) in semisolid cultures is much higher than that required to stimulate BFU-E in the human bone marrow *in vivo* [37]. Similarly, higher concentrations of other haematopoietic growth factors are required to elicit a response in target cells in methylcellulose compared with bone marrow *in vivo*. We have also recently identified (R. S. Verma, A. R. Unune & A. C. Antony, unpublished work) the presence of S-FBPs in conditioned medium (used in methylcellulose media), which could also bind and sequester some PteGlu from the EFC, resulting in less free PteGlu available for cellular uptake during erythropoiesis *in vitro*.

Previous experiments *in vivo* and *in vitro* also suggest that passive diffusion may be the predominant operative mechanism for PteGlu uptake at high EFC. When supra-pharmacological doses of PteGlu were administered to non-folate-deficient human subjects, there was significant PteGlu uptake (up to 11-fold higher) by erythrocytes [38]. The net intracellular PteGlu was, however, greater than concomitant serum PteGlu levels, which was due to rapid clearance of serum PteGlu [39] and/or interaction of PteGlu with haemaglobin [40,41]. Cooper & Peyman [42] also showed RBC-ghost permeability to PteGlu and its pentaglutamate form without chemical modification at high EFC, and DaCosta & Iqbal [27] reported that, although MTX uptake by human RBC *in vitro* was absent at an EFC (MTX) up to 1 μM, uptake of MTX substantially increased above 10 μM by a mechanism that was not saturated even at 1 mM.

Functional intracellular P-FBPs in human KB cells [1–3,9,10] have similar affinity for MTX polyglutamates as the physiological PteGlu species, namely 5-CH_3H_4PteGlu [5]. It is therefore significant that high EFC (of PteGlu) in the growth media of cultured human KB cells profoundly influenced MTX metabolism [9]. Previous studies [43–45] have likewise shown that the EFC (of MTX) influences the formation of MTX polyglutamates intracellularly, a fact that is of importance, since polyglutamation enhances cytotoxicity [46]. Taken together, these data underscore the functional significance of the extracellular as well as the intracellular PteGlu concentration in modulating the effect of MTX in human cells *in vitro*. A clinical correlate is the fact that the steady-state EFC (of MTX) is of major importance in the ultimate outcome in patients with acute lymphocytic leukaemia [47]. Therefore, if passive PteGlu-uptake process is operative physiologically, or can be exploited pharmacologically, the modulatory role of the pre-existing serum level in determining the efficacy and toxicity of MTX *in vivo* intracellularly in malignant and normal cells respectively could be of importance in individual patients.

The mechanism of action of high doses of PteGlu in reversing PteGlu deficiency in familial aplastic anaemia with a defect of cellular PteGlu uptake [48] and congenital PteGlu malabsorption syndromes [49] has not been elucidated. If affected cells (haematopoietic precursors and gastrointestinal cells respectively) have an acquired numerical deficiency or functional abnormality in their PteGlu-transport proteins, the reversal of PteGlu deficiency (by supra-pharmacological doses of PteGlu) may be due to intracellular accumulation of PteGlu by the non-specific PteGlu-uptake mechanism. The recognition of conditions whereby non-specific PteGlu uptake is prominent has significant bearing on the design of studies which specifically assess FBP-mediated PteGlu uptake by intact cells whose functional PteGlu transport proteins are up- or down-regulated by the EFC [11,50]. The (inadvertent) use of high EFC (of radioligands) in uptake studies and determination of total PteGlu uptake alone would not give any indication of the degree of regulation of FBPs on intact cells unless specific PteGlu binding and uptake were distinguished from non-specific uptake mechanisms.

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