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FOLIC ACID AND ITS PENTAGLUTAMATE LEAK FROM HUMAN ERYTHROCYTE GHOSTS BUT NOT FROM LIPOSOMES

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Liposomes were impermeable to folic acid (PteGlu) and its pentaglutamate ($PteGlu_5$), but both folates leaked from human erythrocyte ghosts at a similar rate. $PteGlu_5$ leaking from ghosts was recovered in suspending medium. This indicates that although neither folate penetrates phospholipid membranes, both can traverse erythrocyte membranes without chemical modification. Reduced monoglutamate folate entered erythrocyte ghosts more slowly than it exited. Leakage of folate in both directions was slower than that of urate which, unlike folate, did not exit faster than it entered human erythrocyte ghosts.

Mammalian cells accumulate folate by permissive or active transmembrane transport followed by metabolic conversion and synthesis of polyglutamate folates [1,2]. Intracellular polyglutamate folate is retained within cells, but in some cells under certain conditions, some of this material may leak from cells and enter the extracellular medium [1]. Certain reduced folates and 4-NH_2-10-CH_3-PteGlu enter and leave some cells more readily than does PteGlu [3–5]. to determine if folate leaks across cell membranes when unassociated with intracellular binders, and whether this leakage can occur across lipid membranes devoid of transport proteins, we have examined the leaking of PteGlu and of $PteGlu_5$ from human erythrocyte ghosts and from liposomes. We have compared this leakage with entry of folate into intact erythrocytes to determine if exit of intracellular folate from ghosts is due to altered permeability in the ghosts or a property of erythrocyte membranes.

Liposomes were prepared by mixing 4.837 mg of cholesterol, 4.0 mg of dicetyl phosphate (Nutritional Biochemicals Corp.), and 75.2 mg of L-α-phosphatidylcholine derived from egg yolk (Sigma Chemicals Corp.) in 0.5 ml volumes of chloroform and evaporating the mixture to dryness [6]. This was mixed with sealing solution which contained ^{125}I human serum albumen (10^{-10} M) and either PteGlu (10^{-5} M) or $PteGlu_5$ (10^{-6} M) and sometimes human serum albumin ($1.7 \cdot 10^{-5}$ M) in water. After incubation with mixing for 2 h, the solution was sonified for 2 min in an ice bath and filtration and folate was released from liposomes by boiling for 10 min.

Erythrocytes were obtained from fresh heparinized blood drawn from normal volunteers. Cells were washed thrice in ice-cold saline and erythrocyte ghosts prepared as follows: one volume of washed erythrocytes was lysed by pipetting drop-wise with mixing into 20 vol. of lysing solution at 0°C. After 10 min, resealing solution which contained the material to be incorporated into ghosts was added and mixed. The mixture was incubated at 37°C for 45 min to seal membranes. Ghosts were washed in incubation solution at 0°C.

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Lysing solution contained 5 mM $MgCl_2$, 10 mM mercaptoethanol and folate or urate in the concentration indicated in 10 mM Tris-HCl at pH 7.40. Resealing solution contained NaCl or KCl to bring the final concentration in the solution to 0.15 mM.

(±)-5-[^{14}C]CH_3-H_4PteGlu was purchased from Amersham Corporation, and purified by stepwise elution with ammonium bicarbonate from DEAE-cellulose [7], freeze-dried and stored in 50 μg/ml ascorbic acid at −20°C. Unlabeled folates were purchased from Sigma and purified in a similar fashion. $PteGlu_5$ was a gift from Dr. C.M. Baugh of the Department of Biochemistry, University of Southern Alabama. More than 97% of this material was excluded from Sephadex G-25 indicating that it was poluglutamate folate [8]. Microbiological assay utilized *Lactobacillus casei* ATCC 7469 and, commercial assay media (Difco), and slight modifications [9,10] of standard techniques [11]. Folate was extracted from erythrocyte ghosts by boiling for 10 min in 0.01 M potassium phosphate, pH 9.2 and assayed with and without treatment with human folate conjugase. Radioactive counting utilized a liquid scintillation spectrometer (Packard Tricarb), commercial scintillation mixtures (Aquasol, New England Nuclear Corp.) and internal standards to correct for quenching.

As illustrated in Table I, when PteGlu or $PteGlu_5$ were incorporated into liposomes with ^{125}I-labeled human serum albumin, the ratio of folate: ^{125}I in liposomes excluded from Sephadex G-200 was unchanged after preparation, and after 18 h of subsequent incubation at room temperature. Thus, neither folate leaked from liposomes under these conditions. The lower values for both iodine and folate shown after incubation are caused by dilution of intact liposomes during refiltration through Sephadex G-200. More than 97% of the ^{125}I-labeled albumin remained associated with the liposomes during incubation and refiltration. Folate retention was identical when the study was done using 0.1 μM albumin, or only trace quantities (10^{-10} M). When PteGlu or $PteGlu_5$ were incorporated into human erythrocyte ghosts, considerable loss of PteGlu and $PteGlu_5$ was observed (Table II, Table III). Folate content was assayed microbiologically in washed ghosts and in media, and the latter was corrected for folate entering by hemolysis based on appearance of hemoglobin in the media during incubation (Table II). Of the folate recovered in the media during incubation of ghosts containing $PteGlu_5$, most was polyglutamate in not supporting growth of *L. casei* unless treated with conjugate. $PteGlu_5$ within ghosts did not hydrolyse to monoglutamate, but some hydrolysis of extracellular polyglutamate folate was observed (Table III).

TABLE I

RETENTION OF FOLATE AND ^{125}I-LABELED ALBUMIN IN LIPOSOMES

Preparation	^{125}I (cpm)	Folate (ng)	Folate/^{125}I (ng/10^6 cpm)
PteGlu-liposomes			
separated	26298	47.0	1778 ±234
after incub.	10894	18.25	1675 ±184
$PteGlu_5$-liposomes			
separated	40318	2.5	62.4 ± 9.9
after incub.	15921	1.4	87.9 ± 26.5

To determine if ghosts were more premeable to folate than are intact erythrocytes, ghosts were incubated in 2 μM (±)-5-[^{14}C]CH_3-H_4PteGlu for 4 h and the rate of entry of ^{14}C into washed ghosts and intact erythrocytes was determined. The rate of energy of ^{14}C activity was calculated by the method of Ihler et al. [12], assuming first order kinetics of entry and egress of solute. Rate constants calculated for entry into erythrocytes and ghosts and for egress from ghosts were constant between 1 and 240 min of incubation. Rate constants of entry of this material ranged from 0.01 to 0.05 per hour in different experiments but in studies done simultaneously, were not different in ghosts and in intact erythrocytes ($t_{Student} = 0.710$, $P < 0.05$). Rate constant for entry into ghosts were consistently smaller than (about 50%) for egress ($t = 3.799$, $P < 0.001$), whereas rate constants for entry and egress of urate were similar, and 20–200 times those for the folate. Rate constants for entry and exit of ^{3}H-labeled PteGlu in ghosts was 1/7–1/10 that for 5-CH_3-H_4PteGlu with exit faster than entry.

These studies indicate that whereas liposomes were not permeable to monoglutamate or pentaglutamate forms of folic acid, human erythrocyte

TABLE II

LEAKAGE OF PteGlu FROM HUMAN ERYTHROCYTE GHOSTS IN VITRO

Incubation (h)	Hemolysis (%)	Folate content (ng)				Lost from ghosts (%)
		Ghosts [a]	Media	Hemolysed ghosts	Total	
0	1.04	269	12.1	2.8	285	0
1	1.77	247	24.8	4.7	281	8.9
2	1.81	217	34.4	4.9	261	20.3
4	4.04	205	49.1	10.9	276	27.0

[a] Corrected for hemolysis.

TABLE III

LEAKAGE OF $PteGlu_5$ FROM HUMAN ERYTHROCYTE GHOSTS. POLYGLUTAMATE AND MONOGLUTAMATE IN GHOSTS AND MEDIA

Incubation (h)	Hemolysis (%)	Folate content (ng)				Lost from ghosts [a] (%)
		Ghosts [a]		Media [a]		
		PolyGlu	MonoGlu	PolyGlu	MonoGlu	
0	0.73	75.5	0	3.2	1.0	0
1	2.86	81.9	0	4.0	0	0
2	2.41	66.2	0	8.2	2.75	13.8
4	3.70	57.2	0	10.2	2.75	26.5

[a] Corrected for hemolysis.

ghosts were permeable to both equally. The use of large concentrations of intracellular folate prevented interference which might have been caused by intracellular binders [13] and was far in excess of the remaining endogenous folate of the erythrocyte ghosts used. Most of the polyglutamate folate appearing in the extracellular media was intact polyglutamate indicating that it left erythrocyte ghosts without hydrolysis. The loss of pentaglutamate folate from erythrocyte ghosts was much in excess of hemoglobin recovered in the medium, indicating that is could not have been derived from hemolysed cells. It might have leaked from ghosts permeable to small molecules and not to hemoglobin, but this does not invalidate the conclusion that erythrocyte membranes are permeable to folates. Our failure to observe significant difference in permeability of intact washed erythrocytes and ghosts to a reduced folate supports the physiological relevance of leakage of $PteGlu_5$ from erythrocyte ghosts. We have also observed that folate leaves erythrocyte ghosts more rapidly than it enters [14], unlike urate which traverses such membranes much more rapidly than does folate. This suggests that folate enters and leaves through specialized structures and that these transport pathways are accessible to polyglutamate folate. It suggests that relative impermeability of cell membranes to polyglutamate folate may not explain the selective concentration of polyglutamate over monoglutamate folate within mammalian cells. Certain reduced monoglutamate folates and methotrexate appear to be transported across the membranes of many mammalian cells more rapidly and by a different pathway than does PteGlu. Preferential transport of such reduced folates has been observed in the human erythrocyte [5].

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