Human placental microvilli contain high-affinity binding sites for folate

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Uptake of [^3H]pteroylglutamic acid ([^3H]PteGlu) was studied in microvilli isolated from the syncytiotrophoblast of the human term placenta. The effect of changes in medium osmolality on the equilibrium uptake of [^3H]PteGlu was negligible, which suggested that the observed uptake represented binding to proteins on or within the microvilli rather than translocation of the vitamin from the incubation medium to a free state in the intravesicular fluid. Equilibrium uptake experiments performed over a wide range of [^3H]PteGlu concentrations disclosed a class of binding sites with an association constant of 0.3 nM^{-1} as well as a second class of sites with high capacity and low affinity. Binding of [^3H]PteGlu at the high-affinity sites was inhibited by tetrahydrofolate and N^5 -methyltetrahydrofolate, but not by several other structural analogues. It is likely that the high-affinity binding sites are receptors for maternal plasma folate; however, their role in placental transport or storage of the vitamin was not delineated in these studies.

Little is known regarding the mechanism of transport of vitamins by the human placenta (Chamberlain & Wilkinson, 1979). Because the blood levels of folates are higher in the foetus than in the mother, it has been inferred that active-transport processes are involved (Landon *et al.*, 1975; Strelling, 1976). A particulate folate-binding protein has recently been solubilized and isolated from a homogenate of human term placenta (Antony *et al.*, 1981). Although its subcellular location was not determined, it was suggested that this protein was a folate receptor that facilitated folate transport.

Membrane-associated folate-binding proteins have also been reported in rat liver (Zamierowski & Wagner, 1977), rabbit choroid plexus (Spector, 1977) and in cultured human KB cells (McHugh & Cheng, 1979). Binding and transport of folates have been localized specifically to the plasma membrane in preparations obtained from rat kidney (Selhub & Rosenberg, 1978), rat intestine (Leslie & Rowe, 1972; Selhub & Rosenberg, 1981) and L1210 mouse leukaemia cells (Yang *et al.*, 1979).

The present study was undertaken to gain insight into the subcellular processes involved in folate transport by the human placenta. For this purpose we have studied the uptake of PteGlu by microvilli isolated from the maternal surface of the human term placenta.

Abbreviation used: PteGlu, pteroylglutamic acid.

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Experimental

2-Amino[1- ^{14}C]isobutyric acid (sp. radioactivity 56 Ci/mol) and [$G\text{-}^3\text{H}$]PteGlu (potassium salt; 5 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. They were stored at -20°C and used within 2 months of arrival. According to information supplied by Amersham International, the purity of the [^3H]PteGlu should have declined over the 2 months time period by no more than 4%. Unlabelled PteGlu and folate analogues were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mixed cellulose ester filters (diameter 25 mm; pore size $0.45\text{ }\mu\text{m}$) were obtained from the Millipore Corp. (Bedford, MA, U.S.A.; catalogue no. HAWP 02500).

Microvilli were isolated from the syncytiotrophoblast of the normal human term placenta by a method (Truman *et al.*, 1981) based on that of Smith *et al.* (1974). In the present study, the microvilli were not subfractionated. The microvilli were suspended in Krebs–Ringer phosphate buffer, pH 7.4 (Ruzicky *et al.*, 1978). The isolated microvilli could be stored at 4°C for at least 4 days with no discernible effect on [^3H]PteGlu uptake.

Studies on uptake were performed as follows unless otherwise indicated. A solution ($50\text{ }\mu\text{l}$) of [^3H]PteGlu in Krebs–Ringer phosphate buffer, pH 7.4, and a further $50\text{ }\mu\text{l}$ of the same buffer containing any other chemicals to be added were transferred to glass test tubes ($12\text{ mm} \times 75\text{ mm}$) in a water bath

at 37°C. Incubations were initiated by the addition of 100 μ l of the microvillous suspension (about 500 μ g of protein). In all equilibrium incubations the tubes were capped to minimize evaporation. At the end of the incubation period, 1.8 ml of ice-cold buffer was added, the mixture immediately filtered and the filter was washed twice with 5 ml of the cold buffer. A filtering assembly attached to a water-jet filter pump was used. A control incubation without microvilli was run with many, but not all, sets of experiments. In experiments lacking a control for non-specific binding to the filter, a control value was used that had been obtained from a similar previous experiment. The amount of radioactivity that bound non-specifically to the filters was negligible in most experiments, but became a substantial proportion of the total radioactivity on the filter in experiments in which the uptake by microvilli was very low and the initial amount of radioactivity in the incubations was very high. In all experiments, the value for non-specific binding was subtracted from the observed radioactivity on the filter. The filters were dried overnight at room temperature in uncapped plastic vials. The next morning, 5 ml of a solution of 2,5-diphenyloxazole (0.4%, w/v) in toluene was added and the radioactivity was measured by liquid-scintillation spectrometry. Radioactivity was

measured until the counting error was 2% or less. The radioactivity (d.p.m.) values were estimated by drying known quantities of [3 H]PteGlu on filters that contained unlabelled microvilli and measuring the radioactivity in an identical manner. Efficiency for 3 H was 30–34% and for 14 C was 80–82%.

For trans-stimulation studies, the microvillous suspension was made 200 μ M in N^5 -methyltetrahydrofolate and 100 μ M in β -mercaptoethanol. After incubation for 15 min at 37°C, the mixture was centrifuged (105000g for 30 min at 4°C) and the pellet was suspended in 5 ml of 50 mM-potassium phosphate buffer, pH 7.4, which also contained 100 μ M- β -mercaptoethanol. For control incubations, microvilli were treated in the same way, except that N^5 -methyltetrahydrofolate was omitted. Uptake of [3 H]PteGlu by preloaded and control microvilli was assayed as described above, with 100 μ l of the resuspended microvilli and 5 nM-[3 H]PteGlu in a total incubation volume of 0.2 ml.

The MODFIT computer program together with the MASSACT model (McIntosh & McIntosh, 1980) were used to determine by non-linear regression the association constant for the binding of [3 H]PteGlu to the microvilli.

Protein concentration was measured by a modification (Markwell *et al.*, 1978) of the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

The time course of [3 H]PteGlu uptake by the microvilli is shown in Fig. 1. Essentially no difference was observed in the uptake during the first 30 min of incubation in the presence or absence of an inwardly directed Na^+ gradient. Uptake was slightly greater at 60 min in the presence of the Na^+ gradient, whereas in its absence equilibrium was reached in about 30 min.

Studies were next aimed at differentiating between the binding of [3 H]PteGlu to the microvilli and its translocation from the incubation medium to a free state in the intravesicular fluid. The uptake of 2-amino[14 C]isobutyric acid, a substance known to be transported to the interior of the microvilli *in vitro* (Ruzycski *et al.*, 1978; Fant *et al.*, 1979), was used as a basis of comparison. Some of the studies were conducted in the presence of Triton X-100 or deoxycholate at concentrations that, in rat liver microsomes, induce membrane pores or openings without leading to extensive membrane disassembly (Kreibich & Sabatini, 1974). When the microvilli become leaky, internal contents will be lost during the filter assay and the observed uptake at equilibrium will be less than that in intact microvilli. In contrast, if the uptake

Fig. 1. Time course of uptake of [3 H]PteGlu by placental microvilli in the presence and in the absence of an inwardly directed Na^+ gradient

For full experimental details see the text. To study the effect of an inwardly directed Na^+ gradient (●), the microvilli were prepared in a Krebs-Ringer phosphate buffer that was 154 mM in K^+ instead of Na^+ (Ruzycski *et al.*, 1978). The initial concentration of [3 H]PteGlu was 50 nM. The data are means of duplicate experiments.

Fig. 2. Effects of changes in the osmolality of the incubation medium and the presence of detergents on the uptake of [³H]PteGlu and 2-amino[¹⁴C]isobutyric acid by placental microvilli

For experimental details see the text. The medium contained sufficient sucrose to give the desired osmolality. The microvilli were incubated in the medium (with or without detergent) for 1 min at 37°C before the addition of the substrate (a) 2-amino[¹⁴C]isobutyric acid (50 μM; incubation time 60 min) or (b) [³H]PteGlu (50 nM; incubation time 60 min). Incubations were performed in the absence (○) of detergents and in the presence of 0.03% deoxycholate (●) or 0.1% Triton X-100 (△). The data are means of duplicate experiments.

observed with intact microvilli is due to binding rather than transport, interruption of the integrity of the microvilli should result in little change in the uptake at equilibrium so long as the detergents do not affect the binding directly.

As expected, the uptake of 2-amino[¹⁴C]iso-

Fig. 3. Plot of the ratio bound [³H]PteGlu/total [³H]PteGlu versus bound [³H]PteGlu

Incubations were performed for 90 min. Experimental details are given in the text.

butyric acid in the absence of detergent decreased as the osmolality of the medium increased (Fig. 2a). In the presence of 0.03% deoxycholate, the uptake was decreased to about 42% of control values for most osmolalities, and uptake at all osmolalities was abolished in the presence of 0.1% Triton X-100. Under the same conditions, uptake of [³H]PteGlu differed markedly from that observed for 2-amino[¹⁴C]isobutyric acid (Fig. 2b). Uptake in the presence or in the absence of detergent decreased only slightly as the osmolality of the incubation medium increased and 0.03% deoxycholate was without effect. Uptake in the presence of 0.1% Triton X-100 was 55–65% of the control values.

The uptake at equilibrium over a wide range of concentrations of [³H]PteGlu is shown in Fig. 3. An association constant of 0.3 nM⁻¹ was estimated for the binding of [³H]PteGlu to the microvilli. A curve generated by an equation with three variables fitted the data better than one with two or four variables, and the third term was relatively large. These findings, together with the shape of the curve, suggested the existence of a second binding site with a much lower affinity for folate, but the data were insufficient to determine an association constant for such a site. A Scatchard plot (Scatchard, 1949) of the data (not shown) demonstrated a marked upward concavity, which confirmed the existence of binding sites with different affinities. The results given in Table 1 show that, during the 90 min incubation period used to assay uptake at equilibrium, the receptors were stable and equilibrium was obtained over the entire range of [³H]folate concentrations used.

Because the results of the equilibrium uptake studies indicated that two components contributed to the observed uptake, it was decided to study the uptake at a concentration of [³H]PteGlu (5 nM) at which the high-affinity, low-capacity, component would predominate. It was considered that, in the studies on uptake versus osmolality of the incubation medium performed at an initial substrate concentration of 50 nM (Fig. 2b), changes in uptake by

Table 1. Effect of time and substrate concentration on the uptake of [^3H]PteGlu by placental microvilli
Experimental details are given in the text.

Incubation time (min) Initial concn. ...	Bound [^3H]PteGlu/total [^3H]PteGlu	
	0.1 nM	2 μM
15	0.55	0.023
30	0.63	0.030
45	0.54	
60	0.58	0.035
75	0.53	
90	0.59	0.035
120		0.034

Fig. 4. Uptake of [^3H]PteGlu at low initial concentration by placental microvilli

Initial [^3H]PteGlu concentration was 5 nM. Data are means of duplicate experiments. (a) Time course; (b) the effects of changes in osmolality of the incubation medium and the presence of detergent. Experimental details and an explanation of the symbols are given in the legend to Fig. 2.

the high-affinity, low-capacity, component might have been masked. It was first shown that, at an initial [^3H]PteGlu concentration of 5 nM, equilibrium was reached in 10–15 min (Fig. 4a). Further studies showed no change in uptake at equilibrium with increasing osmolality of the medium in the presence or absence of low concentrations of detergent (Fig. 4b). Uptake was less in the presence of Triton X-100, but was not decreased by 0.03% deoxycholate.

Table 2. Equilibrium uptake of [^3H]PteGlu by placental microvilli in the presence of structural analogues

The methods used to prepare the placental microvilli and to measure the equilibrium uptake are given in the text. The initial concentration of [^3H]PteGlu and of the analogues was 50 nM. In the experiments with tetrahydrofolate and its N^5 -methyl derivative, the incubation medium contained 100 mM- β -mercaptoethanol. At the end of each incubation period (90 min), the ratio bound [^3H]PteGlu/total [^3H]PteGlu was determined. The value for this ratio in an incubation with [^3H]PteGlu alone was designated 0% inhibition and was used as the basis of comparison.

Analogue	Inhibition (%)
Folic acid (unlabelled)	49
N^5 -Methyltetrahydrofolate	18
Tetrahydrofolate	15
N^5 -Formyltetrahydrofolate	0
Amethopterin	0
Adenine	0
<i>p</i> -Aminobenzoylglutamate	0
β -Mercaptoethanol (100 mM)	2

The effects of the presence of various structural analogues on the binding at equilibrium of [^3H]PteGlu to the isolated microvilli are summarized in Table 2. Under the experimental conditions used, only PteGlu, tetrahydrofolate and N^5 -methyltetrahydrofolate decreased the binding of [^3H]PteGlu. It has been suggested (Suresh *et al.*, 1979) that, in L1210 mouse leukaemia cells *in vitro*, folate uptake is mediated by a transport system whose primary substrate is adenine. With isolated placental microvilli *in vitro*, adenine was ineffective as a competitor of either the equilibrium binding of [^3H]PteGlu (Table 2) or its initial rate of uptake (T. Green, unpublished work).

Trans-stimulation of the initial rate of [^3H]PteGlu uptake could not be demonstrated. In incubations with 5 nM-[^3H]PteGlu, the uptake by microvilli previously loaded with N^5 -methyltetra-

hydrofolate was 22% of that observed in control incubations.

Discussion

The results of our studies on [^3H]PteGlu uptake suggest that it represented binding of the vitamin to or within the microvilli rather than its translocation to a free state in the intravesicular fluid. This conclusion is based on our inability to demonstrate a change in the amount of [^3H]PteGlu taken up at equilibrium over a wide range of osmolalities of the incubation medium. Others, using placental microvilli isolated by procedures similar to ours, have observed osmolality-sensitive uptake of glucose (Bissonnette *et al.*, 1981), corticosterone (Fant *et al.*, 1979) and 2-aminoisobutyric acid (Ruzycski *et al.*, 1978; Fant *et al.*, 1979). These results, together with our own using 2-amino[^{14}C]isobutyric acid, show that the volume of the isolated microvilli is sensitive to changes in the osmolality of the external medium.

The effect of low concentrations of detergent on the integrity of vesiculated preparations of placental microvilli has not been reported. In our studies, the uptake of 2-amino[^{14}C]isobutyric acid, a substance known to be translocated from the medium to a free state within the microvilli, was much more severely affected by both Triton X-100 and deoxycholate than was the uptake of [^3H]PteGlu. The results of the detergent experiments can be interpreted in several ways, particularly if a direct effect of the detergent on transport processes is considered as well as a less specific effect on membrane integrity. We believe that the results of the experiments in the presence and in the absence of detergents, when taken together, point to binding of [^3H]PteGlu as the major factor responsible for the uptake observed. The fact that uptake of [^3H]PteGlu was decreased somewhat in the presence of Triton X-100 could mean that the detergent interferes directly with the binding process. It could also mean that a portion of the uptake observed in the absence of detergent is due to transport into the microvilli. If the latter interpretation is correct, then the [^3H]PteGlu must be transported to an osmotically insensitive space within the microvilli.

Other evidence suggesting that the saturable component of [^3H]PteGlu uptake is due to binding and not to translocation was our inability to demonstrate trans-stimulation of the rate of initial uptake of [^3H]PteGlu, which points to the absence of a carrier-mediated shuttle mechanism.

A number of factors could explain our inability to demonstrate translocation of [^3H]PteGlu to a free state within the microvilli. These include the possibility that the transport system was lost or damaged during isolation of the microvilli, the

requisite energy sources were not present in the isolated microvilli, folate is transported only at the crypts between the microvilli or the incubation conditions were not favourable. It is also possible that translocation of [^3H]PteGlu to the interior of the microvilli did occur, but was unrecognized. If the vitamin were bound inside the microvilli, its accumulation would be relatively insensitive to changes in intravesicular volume. Most of these possibilities were not explored. No increase in uptake of [^3H]PteGlu was observed in the presence of an inwardly directed Na^+ gradient under conditions where 2-amino[^{14}C]isobutyric acid uptake by placental microvilli is stimulated (Ruzycski *et al.*, 1978). Leslie & Rowe (1972) also observed binding but not transport of PteGlu in experiments with small-intestinal cells from the rat.

The association constant of 0.3 nM^{-1} that we observed for the saturable component of [^3H]PteGlu uptake by the isolated placental microvilli is less than the value of 3.5 nM^{-1} reported for a putative folate receptor isolated from a placental homogenate (Antony *et al.*, 1981). The difference may in part be due to the different methods used to analyse the binding data (Cornish-Bowden & Eisenthal, 1974; Atkins & Nimmo, 1975; Zivin & Waud, 1982). Antony *et al.* (1981) used a Lineweaver-Burk plot (Lineweaver & Burk, 1934) to calculate the K_a , whereas we used a computer to obtain the K_a by iterative non-linear regression. It is likely that the protein isolated by Antony *et al.* (1981) is responsible for the saturable component of uptake that we observed in the isolated placental microvilli. Presumably this protein facilitates folate transport and thus functions as a receptor, as was suggested. The lack of effect of changes in osmolality on [^3H]PteGlu uptake at high substrate concentration (50 nM) suggests that simple diffusion is not responsible for the non-saturable component of uptake. It may be ascribable to binding that was not saturated with the concentrations of [^3H]PteGlu used. A non-saturable component of uptake of PteGlu in isolated brush-border membrane vesicles from rat intestine was reported (Selhub & Rosenberg, 1981), but was not investigated extensively.

The concentration of total folate in the serum of well-nourished human adults ranges from 14 to 34 nM with N^5 -methyltetrahydrofolate as the major form (Herbert *et al.*, 1962). Most of the folate present in serum is free or loosely bound (Fernandez-Costa & Metz, 1979) and, presumably, is available for placental uptake. The K_a of 0.3 nM^{-1} that we observed for the saturable component of folate uptake by isolated placental microvilli is therefore consistent with reports that the foetal supply of folate may be adequate, at least for haematopoiesis, in the face of moderate maternal deficiency

(Pritchard *et al.*, 1969). However, recent evidence of a possible association between inadequate folate intake by the mother and neural-tube defects in the infant (Laurence *et al.*, 1981) suggests that foetal demands may not always be entirely satisfied, particularly during certain critical periods of development.

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