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The transport of pteridines in CCRF-CEM human lymphoblastic cells*

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Summary. The transport routes used by CCRF-CEM human lymphoblastoid cells for the influx and efflux of unconjugated pteridines were analyzed using [^{3}H]6-hydroxymethylpterin as a model compound. Influx proceeds by a mechanism that exhibits a K_m of 66.7 μM and a V_{max} of 0.077 nmol/min per mg cellular protein. The process is somewhat sensitive to metabolic inhibitors, particularly uncouplers of oxidative phosphorylation, and is significantly affected by the presence of other pteridines in the extracellular medium. The results suggest that pterins with either no 6-substituent (pterin) or those with methyl, hydroxyl, or formyl groups in this position, which exhibit K_i values between 25 and 77 μM, may share the same pathway for uptake. 6-Carboxypterin exhibits low affinity for the system ($K_i > 500\ \mu M$), as do 7-substituted and 6,7-disubstituted derivatives and compounds with larger groups at the 6-position, such as neopterin and biopterin (K_i = 250–300 μM). Efflux of [^{3}H]6-hydroxymethylpterin occurs rapidly and can proceed by at least two routes. The first, comprising approximately 50% of total efflux, is inhibited by extracellular pterins and exhibits similar properties to the uptake system in both its pattern of sensitivity to metabolic inhibitors and its specificity for pteridine structure. The route by which the remaining efflux occurs is relatively insensitive to metabolic inhibition. Adenine significantly inhibits 6-hydroxymethylpterin influx and efflux (K_i = 10.6 μM for uptake) but does not appear to share the same transport system. Similarly, methotrexate and folic acid exhibit little affinity for the unconjugated pteridine transport routes.

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Abbreviations used: HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; BSA, bovine serum albumin; CCCP, carbonyl cyanide m-chlorophenylhydrazone; HEPES buffered saline, 20 mM HEPES, 140 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, adjusted to pH 7.4 with 1 N NaOH; TRIS buffered saline, 20 mM TRIS-HCl, 107 mM NaCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$, 7 mM glucose, adjusted to pH 7.4 with KOH; phosphate-buffered saline (PBS), 138 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, HEPES-sucrose, 20 mM HEPES, 225 mM sucrose, adjusted to pH 7.4 with MgO

Introduction

The transport properties of folates and antifolates have been examined extensively in a variety of bacterial and mammalian cell types [6, 8, 12, 19, 25]; however, the transport properties of pteridines have received little attention. The finding that the compounds of this class with known biological functions (notably, dihydro- and tetrahydrobiopterin) are synthesized intracellularly from GTP [4, 16] has probably lessened the impetus to determine whether a translocation system for unconjugated pteridines exists. However, several recent reports suggest that, at least in certain cell types, these derivatives can be transported efficiently. For example, the breakdown of radioactively labeled folate on storage, which produces unconjugated pteridine products, has been found to generate transport kinetics in murine L1210 cells [9] that differ significantly from those observed with purified folate, apparently due to the significant contribution of the pteridine contaminants (particularly 6-hydroxymethylpterin) to cellular radioactivity uptake. These results concur with direct investigations showing the existence of an active transport system for unconjugated pterins in this cell type [22], a process which is distinct from the mechanism of uptake of folates and antifolates. An active transport system for biopterin has also been described in the microorganism *Crithidia fasciculata* [17, 18], although the existence of a growth requirement for unconjugated pteridines in this species suggests that its transport system may differ from those in cells that can synthesize these compounds intracellularly.

The transport of unconjugated pteridines in human cells has not been directly examined, although there is evidence to suggest that these compounds are excreted and perhaps taken up by certain cell types in ways that can be altered in several disease states. For example, both neopterin and biopterin are excreted in man, where various neoplasias [2, 7] and viral infections [11] have been shown to result in marked elevations in urine and serum levels. Cultured tumor cells have been shown to excrete higher levels of 6-hydroxymethylpterin than normal cells [21], and modulation of human T-lymphocyte activation [24] has been reported for a variety of pteridine derivatives, although it is not known whether this results from interactions at the cell surface or from uptake.

Intracellular unconjugated pteridines can originate by at least two routes. In the first instance, derivatives of biopterin and neopterin can be synthesized from GTP. Reduced derivatives of the former compound are involved as

cofactors in aromatic amino acid hydroxylations [1, 3, 15] in certain tissues, and dihydroneopterin triphosphate is an intermediate formed during biopterin biosynthesis. The second possible source includes those pteridines that could arise from the breakdown of either folates or biopterin and neopterin; these include pterin and its 6-formyl, 6-carboxyl, 6-hydroxymethyl, and 6-hydroxyl derivatives. It has been suggested [13] that transport mechanisms may be necessary primarily to rid the cell of these compounds rather than for the purpose of uptake.

The current studies using the human lymphoblastoid cell line CCRF-CEM were undertaken to investigate the transport of unconjugated pteridines using labeled 6-hydroxymethylpterin as a model compound. The investigation confirmed the existence of a transport system mediating both influx and efflux that is distinct from that used by folates and antifolates.

Materials and methods

Materials. Chemicals were obtained from the following commercial sources: [^{3}H]sodium borohydride (1.4 Ci/mmol), ICN; [2-^{14}C]folic acid (50 mCi/mmol), Amersham; HEPES, DEAE-Sephadex, Sigma; unlabeled pteridines, Dr. B. Schircks Laboratories, Switzerland; fetal calf serum, RPMI-1640 medium, and glutamine, Flow Laboratories; Budgetsolve, Research Products International Corporation.

[^{3}H]6-Hydroxymethylpterin. [^{3}H]6-hydroxymethylpterin was prepared from 6-formylpterin using [^{3}H]sodium borohydride by an adaptation of the method of Thijssen [23]. 6-Formylpterin (5.25 mg dissolved in 0.5 ml 0.1 *N* NaOH) was treated with 0.86 mg [^{3}H]sodium borohydride (1.4 Ci/mmol) by slow addition over 30 min, and the reaction mixture was incubated at room temperature in a dark environment for 2 h. Nonreacted borohydride was oxidized by the addition of 1 *N* HCl and the pH of the reaction mixture was raised to 9.0; the resulting solution was then chromatographed on DEAE-Sephadex (2.5 × 15 cm), which was equilibrated with 0.1 *M* NH_4HCO_3 (pH 7.8) and eluted with the same buffer. The primary UV absorbing peak fractions were pooled and lyophilized. [^{3}H]6-Hydroxymethylpterin (sp. act., 0.41 Ci/mmol) was recovered in 84% yield and was >99% pure by HPLC analysis.

[^{14}C]6-Formylpterin. [^{14}C]-labeled 6-formylpterin was prepared from [2-^{14}C]folic acid (123 μCi/mg, 0.41 mg) diluted to 4 mg by the addition of unlabeled folate and cleaved with 30 μl Br_2 in 100 μl 48% HBr [23]. [2-^{14}C]6-Formylpterin (sp. act., 5.97×10^6 cpm/pmol) was obtained in 53% yield.

HPLC analysis. Pteridines were analyzed using a Beckman Altex HPLC and LDC/Milton Roy fluorescence detector. Separation was achieved on a 0.46 × 25 cm Altex C_{18} reverse-phase column by isocratic elution at 0.8 ml/min using 5% methanol/water.

Growth of cells. CCRF-CEM cells were propagated in RPMI-1640 medium containing 5% heat-inactivated fetal calf serum and 1 m*M* glutamine and maintained at 37° C in a humidified 5% CO_2 atmosphere. Large-scale (100 ml) cultures were grown for transport experiments and harvested in the logarithmic phase of growth.

Measurement of pteridine uptake. Pteridine transport was measured at 37° C or 27° C in HEPES buffered saline unless otherwise stated, and the osmolarity of all buffers was adjusted to 280 mosmol. Individual transport measurements were made on cells collected from logarithmically growing cultures by centrifugation for 10 min at 250 *g*; cells were then washed twice with HEPES buffered saline and suspended for use in the same buffer at 1.2×10^7 cells/ml. Duplicate measurements were made on 0.5-ml aliquots of the cell suspension after preincubation for 1 min at 27° C or 2 min at 37° C to ensure temperature equilibration before the addition of radioactively labeled pteridine and test compounds where indicated. [^{3}H]6-Hydroxymethylpterin uptake was measured using material of sp. act. 3.07×10^5cpm/nmol at a final concentration of 50 μ*M* in each assay. Cells were incubated with shaking at 27° C for 45 s unless otherwise indicated, and the reaction was stopped by the addition of 7 ml ice-cold HEPES buffered saline. Cells were centrifuged at 250 *g* (10 min) and the supernatant was removed by aspiration, after which the cell pellets were washed by resuspension in a second 7-ml aliquot of buffer at 4° C and recovered by centrifugation as described above. The cell pellets were finally suspended in 200 λ buffer and the amount of radioactivity incorporated was estimated by liquid scintillation in 4 ml Budgetsolve. Measurements were corrected for binding in controls incubated at 4° C and transport was expressed as pmol uptake/mg cellular protein. Transport measurements using [^{14}C]6-formylpterin were carried out on cell suspensions incubated for 3 min in the presence of varying concentrations of the label, increased incubation times being necessary to achieve adequate labeling due to the relatively low specific activity of this material. K_m and K_i values for substrates and inhibitors were determined by Lineweaver-Burke and Dixon plots, respectively, and analyzed by linear regression. Protein was measured by the Biuret reaction using BSA as a standard.

Efflux measurements. Cells were harvested as for uptake measurements and preloaded with labeled pteridine by incubation at 37° C for 15 min in the presence of 50 μ*M* [^{3}H]6-hydroxymethylpterin. Cells were cooled to 0° C, centrifuged (250 *g*, 10 min) and washed twice with 8 ml HEPES buffered saline at 4° C to remove extracellular substrate. They were then suspended at 1.2×10^7 cells/ml and divided into 0.5-ml aliquots, each containing 6×10^6 cells for individual measurements. Efflux was determined at 27° C after the addition of test compounds, where indicated, by measurement of the radioactivity associated with the cells following a 2-min incubation with shaking. Efflux was stopped after this time by dilution with 7 ml ice-cold HEPES buffered saline, and the residual radioactivity was determined by liquid scintillation after washing and processing the cells as described above for uptake measurements.

Analysis of the intracellular pool of [^{3}H]6-hydroxymethylpterin. Cells (2.12×10^8) were preloaded with [^{3}H]6-hydroxymethylpterin during a 20-min incubation at 37° C in the presence of a 50 μ*M* concentration of the radiolabeled compound (sp. act., 2.57×10^5cpm/nmol). Unbound material was removed by two washes at 0° C with 10 ml HEPES buffered saline, and the cells were then lysed by three freeze-thawing cycles. The membrane and supernatant

fractions were separated by centrifugation at 16,000 *g* for 45 min, and the radioactive content of the pellet and supernatant was estimated by liquid scintillation. The latter fraction was then subjected to the acid oxidation procedure of Fukushima and Nixon [5] and the resulting eluents from Dowex H^+ chromatography were analyzed by HPLC as described above. The effluent was collected in 0.4-ml fractions and monitored for fluorescence and radioactive content. The specific activity of [^{3}H]6-hydroxymethylpterin recovered from the cell supernatant was estimated by quantifying the pterin in the HPLC eluent using a Beckman model 427 integrator and external standards containing known quantities of pure 6-hydroxymethylpterin.

In separate experiments, 1×10^8 cells were preloaded with [^{3}H]6-hydroxymethylpterin, lysed in three freeze-thawing cycles, then separated into pellet and supernatant fractions by centrifugation as described above. The soluble component was then analyzed by chromatography on a 1.2×28 cm column of Sephadex G-75 equilibrated and eluted with 0.05 *M* potassium phosphate buffer (pH 7.0); fractions (1 ml) were analyzed for absorbance at 280 nm and radioactivity. Separate chromatographic runs under the same conditions were used to establish the elution volume of [^{3}H]6-hydroxymethylpterin alone and protein molecular weight standards comprising 0.5 mg BSA (mol. wt. 66,000) and 0.5 mg cytochrome C (mol. wt. 12,400).

Results

General characteristics of 6-hydroxymethylpterin uptake

The time dependence of [^{3}H]6-hydroxymethylpterin uptake in HEPES buffered saline at 37° C and 27° C is shown in Fig. 1. Influx was extremely rapid at both temperatures. An initial linear phase of uptake lasting less than 1 min was seen at 37° C; it increased to a plateau of 0.32 nmol/mg protein after 20–30 min. Uptake at 27° C was more gradual; the initial linear phase lasted approximately 2 min and was followed by steadily slowing uptake to a plateau of 0.28 nmol/mg protein after 40 min. Using a cell volume of 4×10^{-10}ml/cell [10] and a mean protein content of 1.87×10^{-7}mg/cell, determined by Biuret measurement, the concentration ratio at the steady state between the intracellular 6-hydroxymethylpterin level and that present in the extracellular medium was calculated. With an extracellular pterin concentration of 50 μ*M*, the calculated steady-state ratio was 3:1 at 37° C and 2.6:1 at 27° C, indicating that at both temperatures [^{3}H]6-hydroxymethylpterin was transported by CCRF-CEM cells in a concentrative manner. The possibility that the radioactive pterin was merely binding at the cell surface was considered unlikely, since the labeling in 4° C controls (Fig. 1) represented only 4%–5% of that observed in the cells incubated at 27° C or 37° C. However, this question was further investigated in experiments designed to establish whether [^{3}H]6-hydroxymethylpterin was sequestered or metabolized in a manner that might affect uptake or efflux.

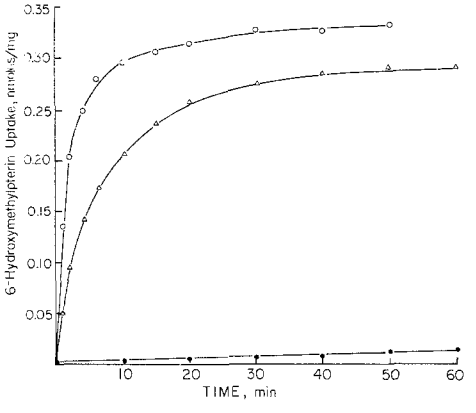

Fig. 1. The influx of [^{3}H]6-hydroxymethylpterin into CCRF-CEM cells as a function of time. Measurements were made in HEPES buffered saline at 27° C (△) or 37° C (○) in the presence of 50 μ*M* [^{3}H]6-hydroxymethylpterin, which was added after a preincubation of 1 min at 27° C or 2 min at 37° C to achieve temperature equilibration. Uptake (nmol/mg protein) was corrected for the incorporation into controls incubated at 4° C for the same times (●)

The metabolic fate of the radioactive compound was examined by HPLC analysis of cell extracts that had been allowed to accumulate [^{3}H]6-hydroxymethylpterin during a 20-min incubation at 37° C, as described in Materials and methods. After removal of unbound label by washing (0° C) and lysing the cells by freeze-thawing, 89% of the total cell-associated counts were found in the cell supernatant and 11%, in the pelleted membrane fraction. When the pellet was washed three times with 1 ml HEPES buffered saline, a further 75% of the initial counts were recovered, supporting the concept that [^{3}H]6-hydroxymethylpterin is taken up by CCRF-CEM cells, not merely bound to the external cell surface. The fate of the [^{3}H]6-hydroxymethylpterin in the soluble fraction was analyzed after oxidation with acidic iodine and separation on HPLC as described in Materials and methods, using the method of Fukushima and Nixon [5]. Recovered pteridines were detected fluorometrically and identified by comparison with known standards. One major peak and four or five minor components were identified; the major peak was the only fraction containing radioactivity, exhibiting the same retention time as authentic 6-hydroxymethylpterin (22 min) as well as chromatographing with this compound as a single peak when cell extracts were mixed with the standard. In two separate experiments, 86% and 95% of the radioactive content of the cell supernatant was recovered in the form of 6-hydroxymethylpterin, indicating that no significant metabolism of this compound occurs. The specific activity for the [^{3}H]6-hydroxymethylpterin recovered after HPLC was found to be 2.79×10^5cpm/nmol by using the procedure outlined in Materials and methods. This value is close to the specific activity of the starting material incubated with the cells (2.57×10^5cpm/mol), further supporting the conclusion that intracellular metabolism of 6-hydroxymethylpterin did not occur during the course of the uptake experiment.

The possibility that [^{3}H]6-hydroxymethylpterin might be bound intracellularly was also investigated by the analysis of cell extracts by gel filtration on Sephadex G-75. The results are shown in Fig. 2. Chromatography of the extract obtained from 1×10^8 cells by freeze-thawing gave a profile containing five broad peaks. Radioactivity was detected in only one of these, which corresponded to the point where a standard of pure [^{3}H]6-hydroxymethylpterin was recovered when it was chromatographed alone under the same conditions. Protein molecular weight standards comprising BSA (mol. wt. 66,000) and cytochrome C (mol. wt. 12,400) eluted significantly earlier from the column than did the radioactivity in the cellular extract, indicating that

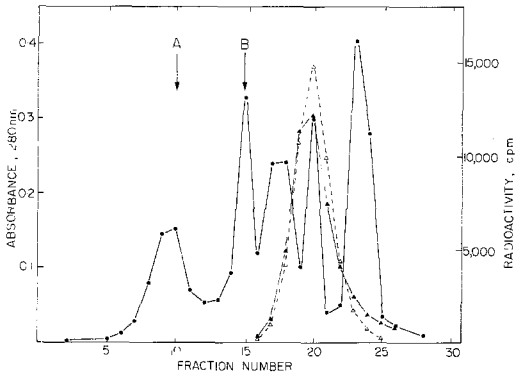

Fig. 2. The elution profile obtained on Sephadex G-75 after application of the soluble supernatant from 1×10^8 CCRF-CEM cells prelabeled with [^{3}H]6-hydroxymethylpterin (50 μM) during a 20-min incubation at 37° C. Excess label was removed by washing at 4° C and the cells were lysed by three freeze-thawing cycles. Membranes were removed by centrifugation and the supernatant was applied to a 1.2×28 cm column of the resin equilibrated and eluted with 0.05 *M* potassium phosphate buffer (pH 7.0). Fractions (1 ml) were analyzed for 280 nm absorbance (●———●) and radioactivity (▲———▲). Separate experiments carried out under the same conditions were used to determine the elution pattern of [^{3}H]6-hydroxymethylpterin alone (△-----△) and molecular weight standards comprising 0.5 mg BSA (*A*) and 0.5 mg cytochrome C (*B*)

the 6-hydroxymethylpterin was probably unbound in the intracellular matrix.

In view of these results, the characteristics of the transport system were investigated in greater detail. Because of the extremely rapid uptake observed at 37° C, measurements of initial rates at this temperature were subject to inaccuracy; therefore, the majority of subsequent analyses were carried out at 27° C. Cells were preincubated for 1 min to achieve temperature equilibration and uptake was measured for 45 s during the linear phase of uptake.

The initial rate of uptake with varying concentrations of [^{3}H]6-hydroxymethylpterin and the analysis of this data by a double reciprocal plot are shown in Fig. 3. A K_t value for half maximal influx of 66.7 μM was determined for this process, together with a maximal velocity (V_{max}) of 0.077 nmol/min per mg cellular protein. A similar experiment was also carried out at 37° C, whereby cells were preincubated for 2 min and uptake was measured for 30 s over the same concentration range of 6-hydroxymethylpterin. Analysis of the data (not shown) gave a K_t value of 29 μM and V_{max} of 0.095 nmol/min per mg protein for uptake at this temperature.

The effect of varying buffer compositions on 6-hydroxymethylpterin uptake was compared using buffers employed in previous studies that examined the transport of folate and antifolate compounds [10, 20]. The results showed that transport was reduced in either TRIS or phosphate-buffered salines to 73% and 75%, respectively, of that observed with HEPES buffered saline and was also depressed to 85% of the control value in HEPES buffer without added anions (HEPES-sucrose). Influx in HEPES buffered saline was enhanced < 10% by the presence of 5 m*M* glucose when measured over an extended period (10 min); however, the initial uptake rate was unaffected. Therefore, transport measurements were primarily made in HEPES buffered saline unless otherwise stated.

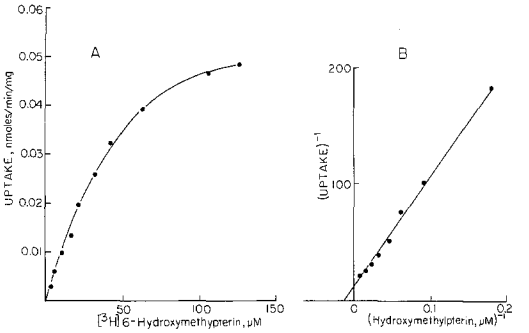

Fig. 3. A: The uptake of varying concentrations of [^{3}H]6-hydroxymethylpterin into CCRF-CEM cells. Measurements were made at 27° C after a 45-s incubation with the indicated concentrations of label and were corrected for the incorporation observed in controls incubated at 4° C; uptake is expressed in nmol/min per mg cellular protein. **B**: Double reciprocal plot of the data in Fig. 2, *A*

Inhibition of 6-hydroxymethylpterin influx

The inhibition of [^{3}H]6-hydroxymethylpterin influx produced by increasing concentrations of unlabeled 6-hydroxymethylpterin in the extracellular medium is shown in Fig. 4. The concentration necessary for half maximal inhibition (K_i) calculated from this data was 52 μM, a value similar to the K_m of 66.7 μM obtained by direct uptake measurements using the labeled compound alone.

A variety of related 6-substituted pterins were tested for their ability to inhibit 6-hydroxymethylpterin uptake; the K_i values determined for these compounds are listed in Table 1. Pterin itself showed the greatest inhibitory properties (K_i = 26 μM), whereas methyl, formyl, or hydroxyl group substitution at position 6 gave K_i values of 70–80 μM. However, the presence of a carboxyl group produced almost no inhibitory properties (K_i > 500 μM). 7-Substituted and 6,7-disubstituted compounds were also poor inhibitors (K_i = 200–300 μM), as were those with larger substituents at the 6-position, such as neopterin, biopterin, and tetrahydrobiopterin.

The uptake of 6-formylpterin was directly examined using the [^{14}C]-labeled compound prepared by cleavage of [2-^{14}C]-folic acid as outlined in Materials and methods, and a concentration for half maximal uptake (K_m) of 100 μM was determined. The similarity of this figure to the

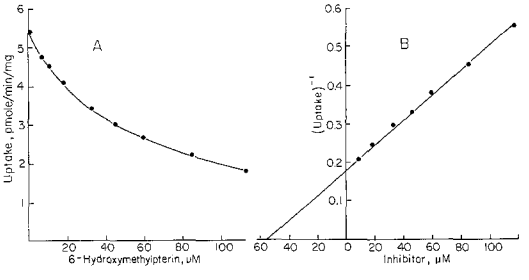

Fig. 4. A: The effect of varying concentrations of unlabeled 6-hydroxymethylpterin on the uptake of a constant concentration (5.4 μM) of the [^{3}H]-labeled derivative. Initial uptake over 45 s was measured in HEPES buffered saline at 27° C following a 1-min temperature preincubation, after which labeled and unlabeled derivatives were added simultaneously. **B:** Dixon plot of the data in Fig. 3, *A*

Table 1. The inhibition of [^{3}H]6-hydroxymethylpterin uptake in CCRF-CEM cells by pteridine derivatives

Pterin	K_i (μM)	Maximum inhibition (%)
6-Hydroxymethylpterin	52	100
6-Methylpterin	74	87
6-Formylpterin	77	95
6-Carboxypterin	>500	–
7-Carboxypterin	275	–
6,7-Dimethylpterin	290	–
6-Hydroxypterin (xanthopterin)	80	87
7-Hydroxypterin (isoxanthopterin)	208	91
Pterin	26	91
Biopterin	253	–
Neopterin	291	–
Biopterin-H_4	269	–

K_i values were calculated from Dixon plots of influx at 50 μM [^{3}H]6-hydroxymethylpterin in the presence of varying amounts of the indicated pterin derivatives. The percentage of total influx inhibitable at infinite concentrations of pterin was calculated by the method of Inui and Christensen [14] from the intercept of plots of the inverse of influx remaining vs the inverse of inhibitor concentration

K_i value assessed for inhibition by unlabeled 6-formylpterin in competition for [^{3}H]6-hydroxymethylpterin uptake (77 μM) suggests that the formyl derivative, and perhaps the other effective inhibitors tested, may be substrates for the same transport system as 6-hydroxymethylpterin itself. This possibility was further explored by analysis of the inhibition produced by these compounds, using the method of Inui and Christensen [14] to define the magnitude of the portion of 6-hydroxymethylpterin uptake that is subject to competitive inhibition by each compound. The inverse of the fraction of uptake remaining was plotted against the inverse of inhibitor concentrations (Fig. 5) and the intercept used to calculate the part of the uptake subject to inhibition; an intercept of 1.0 corresponds to a totally inhibitable process and implies a shared transport system. The results of these analyses are shown in Table 1. Compounds exhibiting high K_i values could not be accurately analyzed by this method due to the low levels of inhibition produced, but analysis of the remaining compounds showed that all of them inhibited uptake (between 87% and 95%), which strongly supports the concept that they are all substrates for a single uptake system.

Since adenine was found to be a strong inhibitor of 6-hydroxymethylpterin uptake in murine L1210 cells [9], it was also tested in the human CCRF-CEM system and found to be a strong inhibitor of uptake (K_i = 10.6 μM). However, analysis of the type described above (Fig. 5) showed that only 66% of the total [^{3}H]6-hydroxymethylpterin uptake could be inhibited by this compound, suggesting that, unlike pterin analogs, it does not share the same transport system. Adenine uptake is also known to be coupled to its conversion to AMP by means of PRPP-dependent adenine phosphoribosyl transferase, and the levels of PRPP are affected by a variety of sugars. However, when 6-hydroxymethylpterin uptake was measured in the presence of various sugars, it was not affected in the same manner as PRPP levels. For example, glucose and inosine, known to elevate PRPP levels, produced no increase in

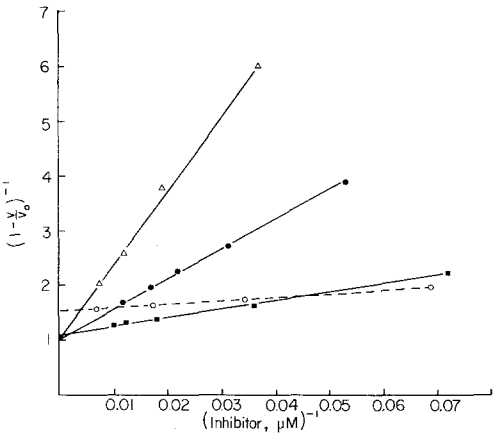

Fig. 5. Double reciprocal plot of the fraction of influx remaining as a function of the concentration of a variety of inhibitors; initial influx rates were measured over 45 s at 27° C. V, observed rate; V_0, uninhibited rate. Inhibitors: ●, 6-hydroxymethylpterin, △, 6-formylpterin; ■, pterin; ○, adenine

pterin uptake; in fact, transport was reduced by almost 50% by 5 mM inosine. Conversely, pterin uptake was unaffected by 5 mM ribose, in which PRPP levels are reduced. Therefore, these results provide additional support for the conclusion that adenine and pterins do not share the same transport system.

A variety of structurally unrelated compounds were also examined for their effects on 6-hydroxymethylpterin transport. The results are shown in Table 2. Uptake was relatively unaffected by azide, oligomycin, and arsenate.

Table 2. The effect of inhibitors on the uptake and efflux of [^{3}H]6-hydroxymethylpterin in CCRF-CEM cells

Addition	Concentration (mM)	[^{3}H]6-Hydroxymethylpterin transport	
		Uptake (%)	Efflux (%)
None	–	100	100
Sodium azide	5	103	103
p-Chloromercuriphenylsulfonate	0.1	93	95
Sodium arsenate	2.0	89	95
2,4-Dinitrophenol	0.001	81	83
Carbonyl cyanide m-chlorophenylhydrazone (CCCP)	1.0	20	68
Ouabain	2.0	68	115
Oligomycin	6 μg/ml	93	98

Uptake was measured at 27°C during a 45-s incubation in HEPES buffered saline with 50 μM [^{3}H]6-hydroxymethylpterin after a 1-min preincubation with the indicated compounds and is expressed as a percentage of control uptake when cells were preincubated alone (0.036 nmol/min per mg protein). Efflux was measured in the same buffer solution after cells were preloaded with label during a 15-min incubation in the presence of 50 μM [^{3}H]6-hydroxymethylpterin. Cells were washed at 0°C and efflux was measured after a 2-min incubation at 27°C either alone (0.09 nmol effluxed/mg protein) or in the presence of the indicated concentrations of putative inhibitors

However, the uncouplers of oxidative phosphorylation, 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone (CCCP), depressed transport; the most effective, CCCP, reduced transport to 20% of that seen in controls, although it exhibited a relatively high K_i of 325 μM. Ouabain depressed uptake to a lesser degree and the mercurial, *p*-chloromercuriphenyl sulfonate, exhibited very slight inhibition.

The effect of the conjugated pterin, folic acid, and the antifolate, methotrexate, which are known to be transported efficiently in this cell type [10], were also examined for their effects on 6-hydroxymethylpterin uptake. Folic acid was not inhibitory at 200 μM levels and methotrexate produced less than a 10% reduction in influx at the same concentration. Since the K_i for inhibition of methotrexate transport by these compounds is 0.9 and 13 μM, respectively, the results suggest that unconjugated pterin transport does not take place by the same route.

Efflux of [^{3}H]6-hydroxymethylpterin

The efflux of [^{3}H]6-hydroxymethylpterin from CEM cells incubated at 37° C and 27° C after preloading with the labeled compound is shown in Fig. 6. Efflux, like influx, was extremely rapid at both temperatures, whereas almost no radioactivity was lost from cells incubated at 4° C. As with uptake measurements, the majority of analyses were carried out at 27° C, where an initial linear phase of efflux could be measured with greater accuracy, data being collected after 2 min efflux in all cases.

The effect of buffer composition on pterin efflux was examined using the same buffers previously described for uptake experiments. Efflux was slowed 10%–15% in TRIS and phosphate-buffered salines and was unaffected by the presence of 1 or 5 mM glucose in HEPES buffered saline or by the replacement of anions by sucrose.

The presence of unlabeled 6-hydroxymethylpterin in the extracellular medium reduced efflux in a concentration-dependent fashion (Fig. 7, *A*); however, efflux was not fully preventable by this means. Analysis by the method of

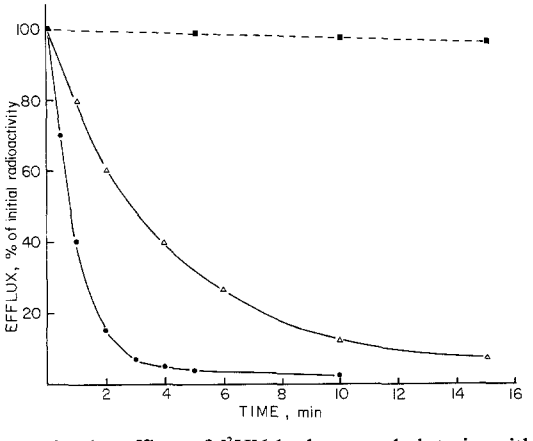

Fig. 6. The efflux of [^{3}H]6-hydroxymethylpterin with time from CCRF-CEM cells incubated at 4° C (■), 27° C (△), and 37° C (●). Cells were preloaded with labeled compound (50 μM) during a 15-min incubation at 27° C, cooled to 4° C and washed to remove unincorporated substrate, then incubated for the indicated times, after which the radioactivity present in the cells was measured. Efflux is expressed as the percentage of labeled derivative remaining; initial loading (100%) was 0.24 nmol/mg

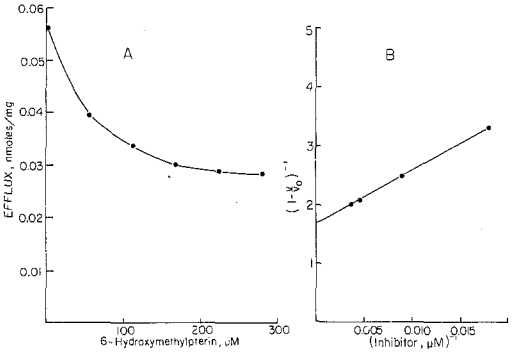

Fig. 7. A: The inhibition of efflux of [^{3}H]6-hydroxymethylpterin from CCRF-CEM cells at 27° C, with varying concentrations of unlabeled 6-hydroxymethylpterin in the extracellular medium. Efflux, expressed as nmol/mg, was measured during a 2-min incubation as described in the legend to Fig. 5. **B:** The inverse of the fraction of efflux remaining as a function of extracellular unlabeled 6-hydroxymethylpterin concentration. V, observed rate; V_o, uninhibited rate

Table 3. The inhibition of [^{3}H]6-hydroxymethylpterin efflux from CCRF-CEM cells by extracellular pteridines

Pterin	K_i (μM)	Effect of 100 μM pterin (% of control)
6-Hydroxymethylpterin	45.0	53.8
6-Methylpterin	48.3	47.8
6-Formylpterin	50.0	63.1
6-Carboxypterin	–	92.6
7-Carboxypterin	–	94.2
6,7-Dimethylpterin	–	85.2
6-Hydroxypterin (xanthopterin)	40.5	47.4
7-Hydroxypterin (isoxanthopterin)	–	92.7
Pterin	15.8	60.1
Biopterin	–	84.2 (200 μM)
Neopterin	–	90.8 (200 μM)

Efflux was measured as described in the legend to Table 2. Inhibition constants (K_i) were measured from Dixon plots of initial efflux rates measured in the presence of varying concentrations of the indicated pterin derivatives. The initial efflux rate observed in the presence of 100 μM pterin (or 200 μM in the case of biopterin and neopterin) is also tabulated as a percentage of the control efflux rate measured in the absence of any extracellular pterins

Inui and Christensen [14], as described earlier for uptake measurements (Fig. 7, *B*); revealed that only 40% of total efflux could be inhibited by 6-hydroxymethylpterin; a K_i value of 52 μM was determined for the inhibitable component. The inhibition constants for the effect of other pterins on efflux are shown in Table 3. Since this constant could only be determined for compounds that exhibited an appreciable effect on efflux, the effect of the presence of 100 or 200 μM concentrations of each compound in the extracellular medium are also tabulated for comparison. The compounds that affected efflux to the greatest extent, namely, pterin and its 6-hydroxymethyl, 6-methyl, 6-formyl, and 6-hydroxyl derivatives, were the same compounds that showed the greatest ability to inhibit 6-hydroxymethylpterin influx (Table 1). Adenine (100 μM) inhibited efflux to 50% of the control value, folic acid had no effect, and methotrexate inhibited the process by 5%–7% at concentrations above 150 μM.

The same range of metabolic inhibitors tested for their effects on uptake were also examined with 6-hydroxymethylpterin efflux (Table 2). The pattern was similar to that seen with influx: azide, arsenate, *p*-chloromercuriphenylsulfonate, and oligomycin had little effect, whereas the uncouplers 2,4-dinitrophenol and CCCP depressed efflux, although the magnitude of the effects were less than those produced on influx. For example, using high levels of CCCP it was shown that approximately 55% of total efflux was not affected by this agent. In contrast to its slight depression of influx, ouabain produced a small enhancement in efflux.

Discussion

A process by which 6-hydroxymethylpterin is taken up into CCRF-CEM human lymphoblastoid cells that is concentrative, saturable, temperature-dependent, and sensitive to inhibition by substrate analogs has been identified. Efflux follows more than one pathway, but approximately 50% occurs by a process sharing the same specificity as the uptake mechanism.

The affinity of a variety of unconjugated pteridines for the 6-hydroxymethylpterin uptake system were examined, and those with either no substituent (pterin) or methyl, formyl, and hydroxyl groups at the 6-position appeared to be likely candidates as alternate substrates for the same pathway. Compounds with more bulky 6-substituents, such as the dihydroxypropyl and trihydroxypropyl groups of biopterin and neopterin, exhibited low affinity, as did 6,7-dimethylpterin and the 7-carboxy and 7-hydroxy derivatives. Compounds with the greatest affinity for the uptake pathway therefore appear to be neutral, unconjugated pterin molecules with relatively small 6-substituents. 6-Carboxypterin is probably a poor substrate because of its anionic form at neutral pH. The observation that uncharged pterin derivatives are the most effective substrates for the uptake process as well as its relative insensitivity to inhibition by metabolic inhibitors suggest that the process may occur by facilitated diffusion.

The existence of a pathway for pterin uptake that exhibits similar specificity has also been described in murine L1210 cells [22]. However, small differences are identifiable; for example, biopterin exhibited a somewhat higher affinity for the 6-hydroxymethylpterin uptake system of L1210 cells (K_i = 180 μM) than that found in the present study using human cells (K_i = 253 μM) and tetrahydrobiopterin showed very poor affinity (K_i = 1.1 mM), whereas in CEM cells the affinity of the reduced compound (269 μM) was comparable with that of the oxidized compound.

Efflux of 6-hydroxymethylpterin, which was not analyzed in detail in the murine cell system, appears to occur by more than one route in CEM cells. The component that could be delineated, comprising approximately 50% of the total, shared properties similar to the influx process. If cellular influx and efflux occur by the same route, it might be expected that the presence of extracellular pterins might stimulate efflux from preloaded cells if the two processes are coupled. However, the observation that pteridine efflux was inhibited under such conditions suggests that it does not occur by an exchange mechanism and that the presence of extracellular pterins may inhibit release by the blockage of common carrier molecules.

Interest in the transport of unconjugated pteridines in vivo centers around observations that certain derivatives are excreted at elevated levels in association with many disorders, particularly malignancies. Some of these compounds have yet to be identified; however, this group is known to include 6-hydroxymethylpterin as well as neopterin, although the source of the latter, often identified by its elevated levels in urine, is unknown. The necessity for an uptake mechanism for these derivatives in vivo has yet to be assessed; however, the transport process described in this report provides evidence that 6-substituted pterins can be released (or taken up) with considerable efficiency at least in this line of human lymphoblastoid cells. However, neopterin and biopterin are somewhat poor candidates for this transport process, which suggests that the origins of excreted 6-hydroxymethylpterin and neopterin in man may differ unless the intracellular levels of neopterin are significantly elevated under some metabolic conditions. Experiments using [^{14}C]- or [^{3}H]-labeled neopterin and biopterin are in progress to assess directly the influx and efflux of these compounds in human cells.

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