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 \( [\text{H}]6 \)-hydroxymethylpterin as a model compound. Influx proceeds by a mechanism that exhibits a \( K_m \) of 66.7 \( \mu M \) and a \( V_{\text{max}} \) 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 \( \mu M \), 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 \( (K_i = 250-300 \mu M) \). Efflux of \( [\text{H}]6 \)-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 \( (K_i = 10.6 \mu M) \) but does not appear to share the same transport system. Similarly, methotrexate and folic acid exhibit little affinity for the unconjugated pteridine transport routes.

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 tetrahydro-biopterin) 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 biotin biosynthesis. The second possible source includes those pteridines that could arise from the breakdown of either folates or biotin and neopterin; these include pterin and its 6-formyl, 6-carboxyl, 6-hydroxymethyl, and 6-hydroxy 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: [3H]sodium borohydride (1.4 Ci/mmol), ICN; [2-14C]folic acid (50 mCi/mmol), Amersham; HEPES, DEAE-Sepharose, Sigma; unlabeled pteridines, Dr. B. Schircks Laboratories, Switzerland; fetal calf serum, RPMI-1640 medium, and glutamine, Flow Laboratories; Budgetsolve, Research Products International Corporation.

[3H]6-Hydroxymethylpterin. [3H]6-hydroxymethylpterin was prepared from 6-formylpterin using [3H]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 [3H]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 [3H]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-Sepharose (2.5 × 15 cm), which was equilibrated with 0.1 M NH4HCO3 (pH 7.8) and eluted with the same buffer. The primary UV absorbing peak fractions were pooled and lyophilized. [3H]6-Hydroxymethylpterin (sp. act., 0.41 Ci/mmol) was recovered in 84% yield and was >99% pure by HPLC analysis.

[14C]6-Formylpterin. [14C]labeled 6-formylpterin was prepared from [2-14C]folic acid (123 µCi/mg, 0.41 mg) diluted to 4 mg by the addition of unlabeled folate and cleaved with 30 µl Br2 in 100 µl 48% HBr [23]. [14C]6-Formylpterin (sp. act., 5.97 × 106 cpm/pmols) 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 C18 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 mM glutamine and maintained at 37°C in a humidified 5% CO2 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 osmolality 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 × 106 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. [3H]6-Hydroxymethylpterin uptake was measured using material of sp. act. 3.07 × 106 cpm/nmol at a final concentration of 50 µM 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 l, 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 [14C]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. Kn and Kc 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 l M [3H]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 × 107 cells/ml and divided into 0.5-ml aliquots, each containing 6 × 106 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 [3H]6-hydroxymethylpterin. Cells (2.12 × 107) were preloaded with [3H]6-hydroxymethylpterin during a 20-min incubation at 37°C in the presence of a 50 µM concentration of the radiolabeled compound (sp. act., 2.57 × 106 cpm/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