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SHORT COMMUNICATIONS

Effects of sulfhydryl inhibitors upon transport of folate compounds into L1210 cells*

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TRANSPORT of folate compounds into L1210 murine leukemia cells is an active, carrier-mediated process.[1-4] Although elucidation of this mechanism will probably require isolation of all the components involved, some preliminary information has been obtained by studying the effects of sulfhydryl inhibitors upon various phases of the transport process. Previous investigators have found that these inhibitors block the uptake of folate compounds into L1210 cells,[1,4,5] and similar results were obtained[6,7] with *p*-chloromercuriphenylsulfonate (pCMS). The latter study, however, revealed a differential effect of the mercurial: pCMS (at 70 μM) completely inhibited the uptake of both amethopterin and 5-methyl tetrahydrofolate, but it had no effect on the uptake of folate. These results demonstrated that folate is transported by a system different from that utilized by the other two compounds, but did not rule out the possibility that separate systems, both sensitive to pCMS, might be responsible for the uptake of 5-methyl tetrahydrofolate and amethopterin.

Further evidence bearing upon this latter question has now been obtained from a more detailed examination of the inhibitory effect of pCMS. For these experiments, L1210 cells were grown at 37° for 48 hr in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin and streptomycin; the final density was 1·0 to 1·5 × 10^6 cells/ml. Cells were collected by centrifugation and suspended in phosphate-buffered saline,[8] pH 7·4, containing the labeled substrate. Initial rates of uptake of 5-methyl tetrahydrofolate and amethopterin were measured under optimal conditions in the presence of various concentrations of pCMS (Fig. 1). Uptake of both compounds was depressed to the same degree as the pCMS concentration increased. Although not shown in the figure, uptake of folate (20 μM) was not inhibited, even when the

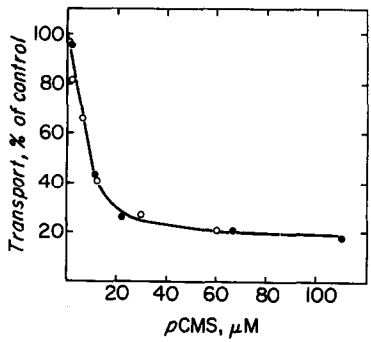

FIG. 1. Effect of pCMS upon transport of 5-methyl tetrahydrofolate (○) and amethopterin (●) into cultured L1210 cells. Cells (5×10^6/ml) were suspended in cold phosphate-buffered saline,[8] pH 7·4, supplemented with 0·11% glucose, and then brought to 37°. At zero time, either [^{14}C]5-methyl tetrahydrofolate (60 mCi/m-mole. Amersham/Searle) or [3′,5′-^{3}H]amethopterin (80 mCi/m-mole, Dhom Products, Ltd.), at final concentrations of 4·2 or 1·9 μM, was added, together with the indicated concentration of pCMS. After 10 min, 2·0-ml samples were withdrawn, added to 9 ml cold 0·9% NaCl and centrifuged. The cell pellets, after being washed with 5 ml 0·9% NaCl, were digested overnight at 37° with 0·6 ml Soluene-100 and then added to 10 ml scintillation fluid (5 g 2,5-diphenyloxazole (PPO) and 0·3 g dimethyl-1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene (POPOP)/liter of toluene). After addition of 0·15 ml glacial acetic acid (to prevent chemiluminescence), the samples were counted at room temperature in a Beckman liquid scintillation counter, LS 233. Uptake was calculated on the basis of nmoles of the labeled compound/10^9 cells. Results are shown as per cent of control, i.e. uptake in the absence of inhibitor.

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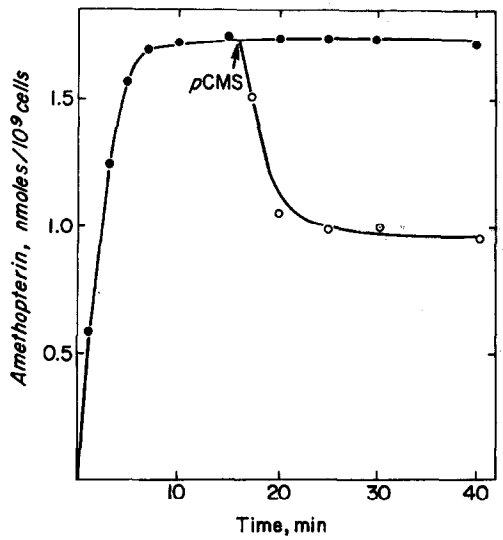

FIG. 2. Effect of pCMS upon steady-state level of amethopterin. The time course of amethopterin transport (●) was measured as described in Fig. 1. In a duplicate experiment, 50 μM pCMS (○) was added at 16 min.

pCMS concentration was 100 μM. These results prove that 5-methyl tetrahydrofolate and amethopterin are transported into L1210 cells via a common, mercurial-sensitive system and that folate transport is mediated by a separate, mercurial-insensitive system. The conclusion that L1210 cells contain two systems for transport of folate compounds is in accord with the results of Nahas *et al.*[4,9] obtained from substrate competition experiments, and is also supported by our observation* that certain amethopterin-resistant L1210 sublines characterized by an impaired transport system for the drug are equally defective in their ability to take up 5-methyl tetrahydrofolate; these mutants transport folate at a normal rate. The concept of two transport systems is at variance, however, with the suggestion of Goldman[3] that folate and amethopterin share the same carrier mechanism, at least in part. Since 5-methyl tetrahydrofolate is the principal folate compound in the circulation of mammals,[10] it is probably the primary substrate for the mercurial-sensitive transport system. Utilization of this system by other reduced folates would be expected from structural considerations. It is surprising, however, that this system should be shared by amethopterin, since the latter is characterized by an oxidized pyrazine ring.

The K_i value of pCMS in the 5-methyl tetrahydrofolate/amethopterin system was 10 μM. Other sulfhydryl inhibitors were less efficient:[6] *p*-chloromercuribenzoate (35 μM); *p*-hydroxymercuribenzoate (45 μM); *N*-ethylmaleimide (460 μM); and iodoacetate (1950 μM). pCMS is not only the most effective inhibitor of this transport system, but it is also reported to be restricted to the extracellular space.[11] At the concentrations used, neither pCMS nor mercaptoethanol (which can reverse the inhibition)[6,7] causes cell destruction or loss of viability.

In addition to distinguishing between the two systems for transport of folate compounds into L1210 cells, pCMS also serves as a probe for the mechanism of the 5-methyl tetrahydrofolate/amethopterin system. This is illustrated by the effect of pCMS on the efflux of amethopterin (the preferred substrate for experiments of this type because it is not metabolized by L1210 cells). As shown in Fig. 2, the uptake of amethopterin is biphasic. The steady-state intracellular level, represented by the plateau in Fig. 2, is the sum of free, exchangeable amethopterin and that which is bound to dihydrofolate reductase. Addition of pCMS causes the steady-state level to fall to 1 nmole/10^9 cells, which is approximately the amount of dihydrofolate reductase present in these cells.* pCMS appears to inactivate the carrier protein at the outer surface of the cell, which allows efflux, but not influx, of the free amethopterin to occur.

In contrast to these results, iodoacetate produces the opposite effect on the steady-state level of amethopterin (Fig. 3). Addition of iodoacetate causes an increase in uptake, indicating that one or both of the intracellular amethopterin pools have been enlarged. Azide[3] and vincristine[12] have been reported previously to produce a similar enhancement of the steady-state level of amethopterin. It is possible that these agents have in common the ability to bring about an increase in the amount of intracellular NADPH.

* R. C. Jackson, D. Niethammer and F. M. Huennekens, manuscript in preparation.

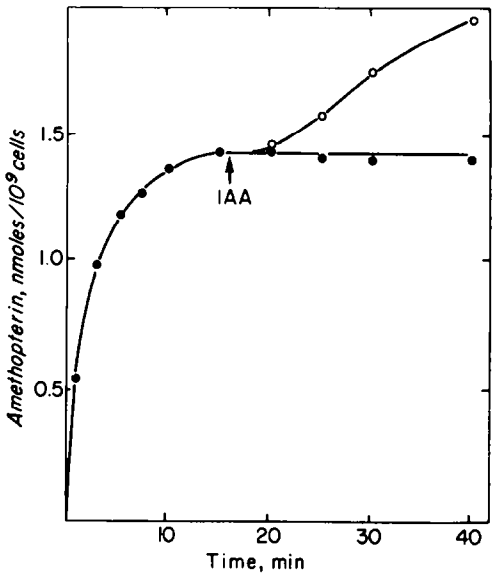

FIG. 3. Effect of iodoacetate (IAA) upon steady-state level of amethopterin. Conditions as in Fig. 2, except that 1·0 mM IAA was added at 16 min.

Since NADPH is known to potentiate binding of amethopterin to dihydrofolate reductase,[13] an increased level of the reduced pyridine nucleotide might lead to an increased amount of enzyme-bound amethopterin. Experiments to test this hypothesis are currently in progress.

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Department of Chemistry,
Scripps Clinic and Research Foundation,
La Jolla, Calif. 92037, *U.S.A.*

JEANNE I. RADER*
DIETRICH NIETHAMMER†
F. M. HUENNEKENS

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* Postdoctoral Fellow of the American Cancer Society. Present address: Department of Biological Chemistry, University of Cincinnati, Cincinnati, Ohio.
† Present address: Department of Pediatrics, University of Ulm, Ulm, Germany.