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STUDIES ON DRUG TRANSPORT BY NORMAL HUMAN LEUKOCYTES*

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Abstract—Transport of the drugs amethopterin, 5-fluorouracil, and 6-mercaptopurine was studied in normal human leukocytes. The data were compared with studies on model compounds (cycloleucine, D-ribose, thiourea) known to be taken up via active transport, facilitated diffusion, or simple diffusion. We found that uptake of 5-fluorouracil and 6-mercaptopurine resembled simple diffusion; these two drugs were also slowly metabolized by cellular enzymes to form nondiffusible nucleotides. Amethopterin was taken up by a process of facilitated diffusion which was saturable, markedly temperature-sensitive, and distinctly nonconcentrative. These data have implications in the design of therapeutic programs.

STUDIES[1–3] have shown that many compounds, e.g. certain amino acids, sugars, and cations, enter mammalian cells by specific, energy-requiring processes. Other compounds either penetrate these cells by simple diffusion (e.g. thiourea) or else, like sucrose and inulin, are excluded. Recent reports show that uptake by human leukocytes of methyl glyoxal-bis(guanylhydrazone)[4] and many amino acids[5, 6] also proceeds by specific energy-requiring processes.

As part of a study of the effects of pharmacologically active agents on human leukocytes, we have investigated drug uptake and exit and the formation of intracellular drug metabolites to seek possible relationships between drug accumulation *in vitro* and drug response *in vivo*. Distinctions between active transport, facilitated diffusion, and simple diffusion were made. This study reports observations on normal human leukocytes; drug uptake by untreated, drug-sensitive and drug-resistant human leukemia cells will be reported separately.

MATERIALS AND METHODS

Blood was collected from 50 normal donors through the plasmapheresis facilities of the Children's Cancer Research Foundation. Leukocytes were isolated by a method based on the procedure of Fallon *et al.*[7] Blood (500 ml) was collected in containers with ACD anticoagulant. The "buffy coat" leukocyte layer was removed and diluted with 1 vol. of a 3% solution of dextran (mol. wt. 177,000) in 0·9% NaCl and allowed to stand at 4° until a distinct separation had taken place. The bottom layer of red cells was discarded, and the leukocyte-rich fraction collected by centrifugation of the supernatant fluid at 150 *g* for 5 min. This and all subsequent operations was carried out at 2–4°. The cell pellet contained 10–20 per cent erythrocytes which were eliminated

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by osmotic lysis in 0·22% NaCl for 30 sec. The use of streptokinase–streptodornase to prevent cell clumping[7] was not found necessary. The leukocytes were collected by centrifugation as before, and resuspended in 9 vol. of incubation medium. The final preparations usually contained 75 per cent granulocytes, 25 per cent lymphocytes, and less than 1 per cent erythrocytes.

Radioactive amethopterin-3′,5′-H^3 (2–9 c/m-mole) was obtained from the Nuclear Chicago Corp.; 6-mercaptopurine-8-C^{14} (25 μc/mg) and thiourea (0·5 mc/m-mole) from New England Nuclear; cycloeucine-1-C^{14} (5 mc/m-mole), 5-fluorouracil-2-C^{14} (20 mc/m-mole), and D-ribose-1-C^{14} (2 mc/m-mole) were from Calbiochem Corp. During storage at −20°, the amethopterin became contaminated with decomposition products and was repurified by chromatography on Whatman 3MM paper with 0·05 M phosphate buffer at pH 7·5. The drug was located by its quenching of the fluorescence of the paper under u.v. light, eluted with water, and concentrated by lyophilization. Other substances were used without purification, although the absorption peak of the 6-mercaptopurine at 320 mμ was occasionally verified.

Dialyzed calf serum was obtained from Grand Island Biological Co., Grand Island, N.Y. TES buffer*[8] was obtained from Calbiochem Corp.

Disposable pipettes (Drummond Microcaps) were used for addition of radioactive substrates to incubation mixtures. Since some batches of these pipettes contained alkaline impurities, all were routinely washed twice with distilled water and dried before use.

Unless otherwise indicated, leukocytes were suspended in 3 parts of buffer and 1 part of dialyzed calf serum hereafter called incubation medium. The pH of this medium at 37° was 7·4. The buffer contained 62 mM TES (pH 7·2), 65 mM NaCl, 15 mM KCl, and 8 mM $CaCl_2$ as suggested by Yunis *et al.*[5] Serum was added to promote stability of leukocytes during incubation. The addition of serum did not interfere with uptake of any substance measured here except amethopterin, which is bound to plasma proteins.[9, 10] The use of Krebs–Ringer bicarbonate with added Tris, as specified by Rosenberg and Downing,[6] yielded data in agreement with results obtained by using the buffer described above.

Each milliliter of the leukocyte suspension used contained 100 mg wet wt. 14–16 mg dry wt. of cells. Data are reported here in terms of accumulation per gram of wet cells.

Incubations of 150 μl of 10% leukocyte suspensions were carried out in 10 × 30 mm siliconized glass tubes at several time and temperature combinations. Solutions of radioactive compounds were added at the beginning of the incubations in volumes of 1–5 μl. The suspensions were shaken in a temperature-controlled oscillating shaker, and incubations were ended by transfer of tubes to an ice bath. After chilling for 1 min with occasional shaking, the cells were collected by centrifugation for 30 sec at 150 *g* in a Misco microcentrifuge and treated by one of three procedures.

Procedure 1. For determinations of drug uptake, cell pellets were washed by resuspension in buffer at 0° to remove extracellular radioactivity, collected by centrifugation, and treated with 250 μl of 0·08 M acetic acid at 60° for 10 min to extract low molecular weight, radioactive compounds.[5] The extracts were clarified by centrifugation for 30 sec at 150 *g*; 200-μl aliquots were used for determination of radioactivity. The amount of intracellular drug removed by washing for 30 sec at 0° may be estimated

* *N*-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid.

from Fig. 4. We estimate that less than 10 per cent of the drug was lost during washing. Negligible amounts of radioactivity were found in the cell debris after the hot acid extraction.

Procedure 2. To measure drug efflux, cell pellets were resuspended in 300 μl of incubation medium and incubated for 5–20 min. After this incubation, the cells were collected and extracted with the hot dilute acetic acid as described above.

Aliquots of 200 μl of the extracts were mixed with 10 ml of a scintillator solution containing, per liter, 600 ml toluene, 400 ml methyl cellosolve, 60 g naphthalene, and 4 g 2,5-*bis*-2-(5-*tert*-butylbenzoxyazoly) thiophene (BBOT). Radioactivity was determined with a Nuclear-Chicago scintillation counter.

Procedure 3. For identification of intracellular radioactive compounds, the cell pellets were resuspended in 10 vol. of 0·5 M $HClO_4$. After 15 min at 0°, the mixture was clarified by centrifugation, and the pH of the supernatant fluid was adjusted by the addition of approximately 17 μl of 1 M KOH. The resulting mixture was chilled, the precipitate of $KClO_4$ was removed by centrifugation, and the fluid was concentrated by lyophilization and used for chromatographic studies.

Alternative procedures. The results reported here were in agreement with data we obtained by Millipore filtration of cell suspensions followed by brief washing.[11] For comparison of the two methods, 5-μ Millipore filters were employed, using gentle (10 cm of water) vacuum. After collection of cells on filter pads moistened with incubation medium, 1 ml of the medium was used for washing to remove extracellular radioactivity, care being taken to avoid drying of the filters. The filter pads containing cells were dissolved in the scintillation fluid described above, and uniformly suspended with the aid of a gelling agent. Since the centrifugation method minimized the danger of breaking fragile cells, this procedure was generally used.

Identification of radioactive intracellular compounds. Lyophilized extracts from procedure 3, described above, were dissolved in a minimum amount of water, and radioactive compounds present were identified by descending paper chromatography on Whatman No. 1 paper. Amethopterin was separated with 0·05 M phosphate buffer at pH 7. An authentic sample of the drug showed an R_f of 0·6. Cycloleucine ($R_f = 0·4$) and thiourea ($R_f = 0·5$) were chromatographed with *n*-butanol–acetic acid–water (4:1:5). No radioactive metabolites of any of these three compounds could be detected by chromatography. A Vanguard 880 Autoscanner was used for detection of radioactivity.

Intracellular metabolites of 5-fluorouracil were separated by paper chromatography with 66% isobutyric acid adjusted to pH 4·6 with ammonia, a mixture previously used for separation of uracil metabolites.[12, 13] We observed 5-fluorouracil ($R_f = 0·6$), 5-fluorouridine monophosphate ($R_f = 0·3$), and 5-fluorouridine triphosphate ($R_f = 0·1$). Radioactive metabolites of 6-mercaptopurine were separated with the butanol–acetic acid–water mixture described above. For 6-mercaptopurine an R_f of 0·8 was found; the major metabolite had an R_f of 0·4. Electrophoretic behavior of these compounds in 0·05 M ammonium formate indicated that the metabolite was 6-mercaptopurine ribotide.[14] Radioactivity was detected with a Vanguard 880 Autoscanner.

RESULTS

In Fig. 1 the time course of uptake of six compounds at 37° is presented. The rapid phase of uptake of all but amethopterin had ended after 15 min.

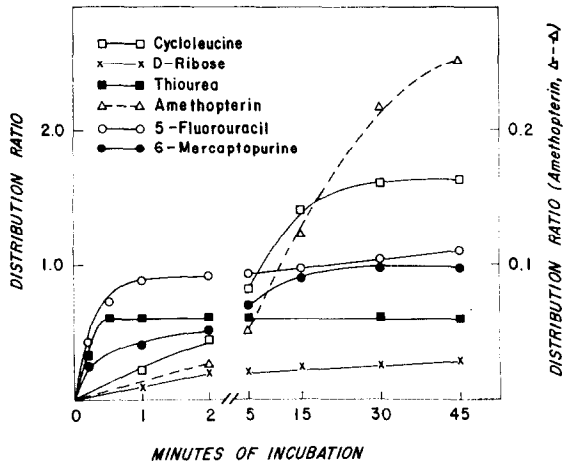

FIG. 1. Uptake by human leukocytes as a time function. Cells were incubated in medium at 37° containing 0·1 mM of radioactive substrates (amethopterin = 0·2 μM). Each point represents the mean of 3–5 replicate determinations run simultaneously. These differed from the mean by no more than $\pm$10 per cent. The drug distribution ratio = moles of drug per gram of cells (wet weight)/mole drug/ml. extracellular fluid.

The effects of variation of temperature upon uptake of test compounds are shown in Fig. 2. An incubation time of 15 min was usually used for these studies, since microscopically observable cell damage and an increase in number of cells permeable to tryptan blue occurred after about 30 min of incubation at 37°. Although entry of all compounds tested was temperature-sensitive over some range, only amethopterin, D-ribose, and cycloleucine entry showed temperature sensitivity over 12°–37°.

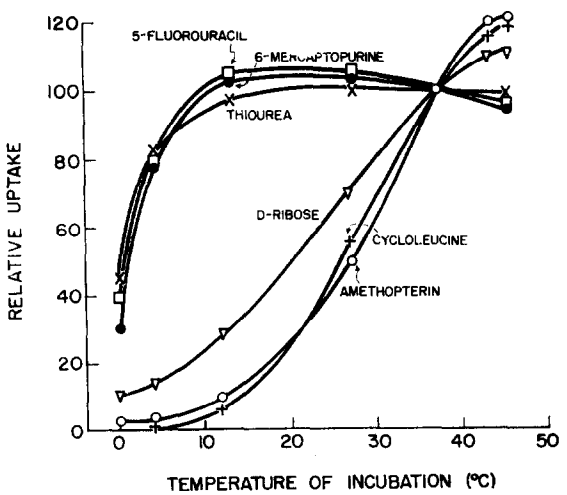

FIG. 2. Temperature dependence of uptake by human leukocytes. Cells were incubated for 15 or 30 min (see Table 1) in medium containing 0·1 mM of labeled substrates (amethopterin = 0·2 μM). Uptake is expressed relative to the level reached when incubation temperature was 37°. Each point represents the mean of 3 duplicate determinations which varied by less than $\pm$10 per cent from the mean.

Uptake of only three of the compounds tested was affected by changes in pH of the medium. These effects are shown in Fig. 3.

Of the compounds tested, only cycloleucine, D-ribose, and amethopterin showed evidence of saturable uptake; the intracellular/extracellular distribution ratios fell as the external concentration of these compounds was raised (Table 1).

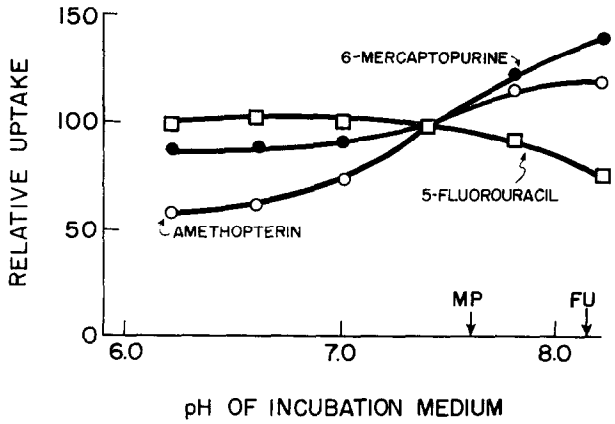

FIG. 3 Influence of pH of incubation medium on uptake by human leukocytes Cells were, incubated for 15 or 30 min (see Table 1) in medium containing 0·1 mM of labeled substrates (amethopterin = 0·2 μM) Data shown here indicate drug uptake relative to the level reached at pH 7·4 the pK values for 5-fluorouracil and 6-mercaptopurine are indicated by arrows Data are shown for a typical experiment; repetitions yielded results which differed from these by less than $\pm$5 per cent.

TABLE 1. DRUG UPTAKE BY HUMAN LEUKOCYTES AS A FUNCTION OF EXTRACELLULAR DRUG LEVEL*

Drug or test compound	Extracellular concentration	Distribution ratio	Time of incubation (min)
Amethopterin	0·2 μM	0·24	
	2·0 μM	0·11	30
	20 μM	0·05	
	200 μM	0·03	
5-Fluorouracil	0·1 mM	0·92	
	1·0 mM	0·91	15
	5·0 mM	0·94	
6-Mercaptopurine	0·1 mM	0·88	
	1·0 mM	0·86	15
	5·0 mM	0·85	
Thiourea	0·1 mM	0·56	
	1·0 mM	0·52	15
	5·0 mM	0·55	
Cycloleucine	0·1 mM	2·0	
	1·0 mM	1·4	30
	5·0 mM	1·1	
D-Ribose	0·1 mM	0·2	
	1·0 mM	0·17	15
	5·0 mM	0·12	

* Cells were incubated at 37° in medium containing specified levels of radioactive compounds and the resulting intracellular/extracullar distribution ratio was measured. Results shown here are averages of 3 determinations which did not differ by more than $\pm$5 per cent.

The rate of loss of these compounds from previously loaded cells is shown in Fig. 4. Efflux of all compounds was temperature-sensitive. Two-phase loss of amethopterin, 6-mercaptopurine, and 5-fluorouracil was observed. The major portions of non-diffusible radioactivity in the cells after incubation with labeled 5-fluorouracil or 6-mercaptopurine were identified by chromatographic procedures as drug nucleotides,

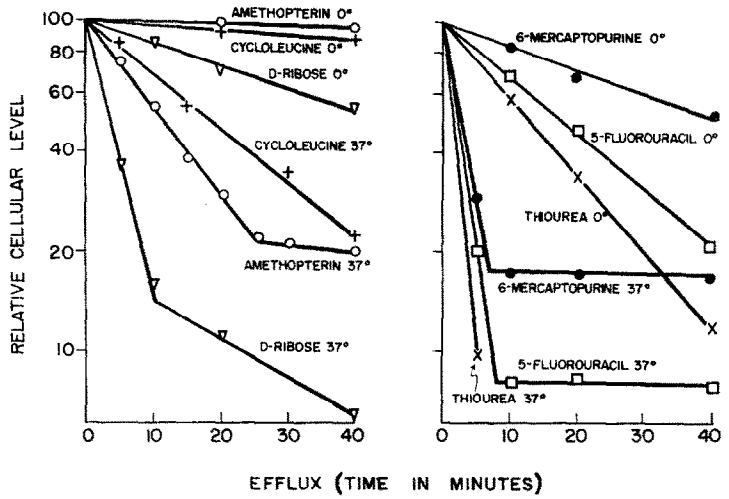

FIG. 4. Exit of labeled substrates from human leukocytes. Cells were incubated for 15 or 30 min (see Table 1) at 37° in medium containing labeled substrates at 0·1 mM (amethopterin = 0·2 μM). Cells were collected, then resuspended in fresh medium at 0° or at 37°, and intracellular levels of substrates were measured at intervals. These levels are shown relative to the intracellular substrate concentrations present at the end of the first incubation. Data are shown for a typical experiment; repetitions yielded results which differed from these by less than ±10 per cent.

as previously described. Conversion of 5-fluorouracil, but not of 6-mercaptopurine, to nucleotide was abolished by addition of 10^{-3} M dinitrophenol to the medium. The slower phase of amethopterin loss from the cells was caused by intracellular drug binding by an enzyme as described later.

DISCUSSION

The methods described here represent procedures we have found suitable for measurement of cellular capacity for transport and metabolism of labeled drugs by human leukocytes, cells from malignant effusions, and dispersions of solid and ascitic tumor cells. Important considerations were maintenance of cell morphology during incubations, absence of cell clumping, and minimal loss of accumulated drug during washing procedures. Times and temperatures of incubations can be chosen to provide data on conditions wherein drug level approximated that achieved during high-dose clinical therapy. These methods should be useful in assessing transport characteristics of new drugs, and in the evaluation of altered transport or accumulation in development of drug resistance.

Compounds tested here were taken up by leukocytes by simple diffusion, facilitated diffusion, i.e. a saturable and/or temperature-sensitive but nonconcentrative process,[15] or by active transport. We have used cycloleucine as a model compound[6] for demonstrating active transport, D-ribose for facilitated diffusion, and thiourea for simple

diffusion.[16] Uptake of cycloleucine was concentrative (Fig. 1), temperature-sensitive (Fig. 2), and showed a saturable component (Table 1). Facilitated diffusion of D-ribose was nonconcentrative, but temperature-sensitive and saturable. Simple diffusion of thiourea was much less affected by temperature changes, and was apparently not saturable. Rates of loss from previously loaded cells were all temperature-sensitive, but at either 37° or at 0°, loss of cycloleucine was slowest and thiourea fastest.

Uptake of 5-fluorouracil and of 6-mercaptopurine closely resembled the simple inward diffusion of thiourea. Uptake of these two drugs was rapid, essentially temperature-insensitive, except near 0°, and apparently not saturable. The gradual increase in uptake of 5-fluorouracil and 6-mercaptopurine shown in Fig. 1 was attributed to cellular formation of nondiffusible drug nucleotides. This would result in an increase in total accumulation of labeled materials by cells. Minor variations in the steady state intracellular–extracellular equilibrium were observed when the pH of the medium was varied (Fig. 3). These variations seem unrelated to drug pK: 5-fluorouracil = 8·15,[17] 6-mercaptopurine = 7·6.[18] The pronounced breaks in the curves showing drug efflux (Fig. 4) were traced to formation of cellular drug nucleotides which were nondiffusible. The observations reported here are in agreement with data reported by Jacquez who found that 5-fluorouracil and adenine, of which 6-mercaptopurine is an analog, freely diffuse into Ehrlich ascites carcinoma cells.[19, 20] Another report, however, suggests active transport of 5-fluorouracil across intestinal epithelium.[21]

If cellular uptake of 5-fluorouracil and 6-mercaptopurine occurs by a process of simple diffusion, 'resistance' to these compounds in cell lines would probably not be based upon altered transport. In the case of 5-fluorouracil, drug resistance in mouse leukemias was found to be related to reduced cellular capacity for drug phosphorylation.[13]

Uptake of amethopterin by leukocytes during a 30-min period was nonconcentrative at all levels treated, but the process was at least partly saturable (Table 1). The striking temperature sensitivity of accumulation of the drug (Fig. 2) together with the demonstration of a saturable component of uptake suggests a process of facilitated diffusion. Uptake of amethopterin was slow; a steady state had not been reached after 45 min of incubation at 37° (Fig. 1). Since this drug was partly bound to serum proteins,[9, 10] omission of horse serum from the incubation medium resulted in a slight elevation of amethopterin uptake. This elevation (not shown here) amounted to a 10–15 per cent increase in both initial uptake rate, and cell accumulation of the drug after 20–30 min.

Additional evidence that suggests facilitated diffusion of amethopterin lies in findings that uptake of the drug by leukocytes could be inhibited by certain amethopterin analogs,[22] which suggests an uptake process of some stereospecificity.

Leukocyte preparations from some patients with amethopterin-resistant leukemias demonstrated impaired capacity for uptake of the drug.[22, 23] A similar impairment of amethopterin uptake was associated with development of drug resistance in animal leukemias *in vivo*[24] and in culture.[25]

In contrast to data reported here, and cited above, Hakala[26] found a slow influx of amethoperin into Sarcoma-180 cells, apparently by a process of free diffusion. She reported that the rate of influx of the drug into S-180 cells was proportional to the external drug level over a broad range, and attributed the observed Q_{10} of 6·2 to a high potential energy barrier to diffusion of the drug molecule across the cell membrane.

The finding here of an apparent saturability of amethopterin transport by leukocytes is the strongest evidence to suggest that these cells have a capacity for facilitated uptake of the drug.

In another report,[27] Jacquez found free diffusion of amethopterin into Ehrlich carcinoma cells, but these experiments were carried out at very high external drug levels, which may have masked a saturable transport process. Evidence has been found to suggest that uptake of amethopterin by Erlich cells is not a process of simple diffusion, when the extracellular drug level is low.*

Clinical relevance. Of the three drugs studied here, amethopterin and its analogs vary widely in clinical effectiveness, depending on size of dose, frequency, and route of administration. The finding that amethopterin, unlike 5-fluorouracil and 6-mercaptopurine, is apparently taken up by facilitated diffusion, may help explain these reports[28, 29] and may provide a basis for recent findings[30, 31] that certain methods of administration e.g. intermittent high doses, may increase the effectiveness of amethopterin.

Uptake of 5-fluorouracil and 6-mercaptopurine appears to be a process of simple diffusion. If these drugs diffuse into cells even at low plasma levels, drug responses might be achieved at low, perhaps nontoxic, plasma levels, as suggested by current clinical studies.[32, 33]

The slow component of amethopterin loss (Fig. 4) was attributed to binding of the drug by cells; tight binding of amethopterin to dihydrofolate reductase, the drug's "target enzyme," has been described.[34] High levels of this enzyme in leukocytes markedly slow amethopterin loss.[35] Delayed loss of 5-fluorouracil and 6-mercaptopurine from pre-loaded cells was traced to formation of drug nucleotides, presumably the pharmacologically active forms.[36, 37] These observations suggest that measurements of both drug uptake and drug loss from human cells could be useful as predictive indices of drug response.

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