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INTRACELLULAR PHOSPHATE AND ITS POSSIBLE ROLE AS AN EXCHANGE ANION FOR ACTIVE TRANSPORT OF METHOTREXATE IN L1210 CELLS[1]

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Summary: L1210 cells transport P_i in the absence of added Na^+. Uptake shows saturation kinetics (K_t = 1.7 mM), is temperature-dependent, and can be reduced 80% by high levels of unlabeled P_i, and thus has the characteristics of a carrier-mediated process. This transport process is also inhibited by methotrexate. The methotrexate-sensitive component constitutes half of total P_i uptake, and is reduced by 50% at a concentration of methotrexate (2 μM) that is comparable to its K_t (1.5 μM) for transport into the cells. An impermeable fluorescent analog of methotrexate and an irreversible inhibitor of the methotrexate transport system (carbodiimide-activated methotrexate) also inhibit this same P_i uptake component. It is concluded that methotrexate and P_i can be transported by the same carrier system. The basis for this shared uptake is suggested to be that the methotrexate carrier protein facilitates the obligatory exchange of extracellular folate compounds for intracellular divalent anions, and that a primary exchange anion is P_i. A principal energy source for active transport of methotrexate might then be the concentration gradient for P_i that is maintained by the Na^+-dependent, P_i transport system of these cells.

L1210 mouse leukemia cells contain a single, high-affinity transport system for the uptake of folate compounds and various folate antagonists, including methotrexate (MTX)[2] (reviewed in Refs. 1 and 2). The process is mediated by a carrier protein that is exposed to the cell surface (3) and is coupled to energy since methotrexate can accumulate within the cells to concentration gradients in excess of 50-fold (4). The fact that various organic and inorganic anions competitively inhibit MTX influx (4-7) and

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[2] Abbreviations: MTX, methotrexate; F-MTX, fluorescein-diaminopentane-methotrexate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Buffer A, 160 mM Hepes and 1 mM $MgCl_2$, pH 7.4 with KOH; and Buffer B, 140 mM NaCl, 10 mM KCl, 20 mM Hepes, and 1 mM $MgCl_2$, pH 7.4 with KOH.

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reduce steady-state levels of the drug (4,6) led to the proposal that MTX enters L1210 cells by an anion exchange mechanism and that gradients of anions act as the energy source for the concentrative uptake of MTX (4-6). The exchange process also appears to be obligatory since efflux of MTX is markedly reduced when anions are omitted from the external medium (8).

A distinguishing feature of MTX transport in L1210 cells is that steady-state levels of the substrate are decreased by glucose or pyruvate (4,9), whereas an increase is observed in the presence of certain metabolic inhibitors (4,9,10). These observations led Goldman to suggest that L1210 cells might contain an ATP-dependent system for pumping MTX out of the cell (5). An alternative explanation is that changes in the metabolic state of the cell might have perturbed intracellular levels of anions (related to ATP synthesis) that exchange for MTX. In the present study, a possible role for P_i in the uptake of MTX was investigated. The results provide evidence that the MTX carrier system can also transport P_i, and, in addition, that the cells maintain a concentration gradient of P_i that would be of sufficient magnitude to serve as an energy source for the uphill transport of MTX.

Materials and Methods

Chemicals. [^{33}P]Ortho P_i in 0.1 N HCl (carrier-free) was purchased from New England Nuclear and was diluted with unlabeled P_i prior to use.

Buffers. L1210 mouse leukemia cells were grown as described previously (11), washed with either Buffer A or Buffer B , and resuspended to a density of 2×10^7/ml. The diameter of L1210 cells suspended in Buffer A or in Buffer B and incubated for 30 min at 37° was a 9.9 ± 0.6 μ and 9.8 ± 0.4 μ, respectively (n = 20). These values were not significantly different from a value of 9.5 ± 0.5 μ obtained with cells incubated similarly in phosphate buffered saline (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 KH_2PO_4, 1.0 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4).

Transport measurements. P_i transport was measured as described previously for MTX (4) in assay mixtures (1.0 ml, final volume) consisting of washed cells (1.6×10^7), the indicated additions, and $^{33}P_i$ (2,500-10,000 cpm/nmole). After incubation (with shaking) for 1 min at 37° (in Na^+-containing buffers) or 10 min at 37° (in Na^+-free buffers), the cells were diluted to 5 ml with 0.15 M KCl, recovered by centrifugation at 1000 x g (5 min, 4°), washed with 8 ml of 0.15 M KCl, resuspended in 1.0 ml of H_2O, and analyzed for radioactivity (4). Uptake of P_i at 4° served as the control. The transport rate (in pmoles/min/mg protein) was calculated from the difference in radioactivity between the experimental and 4°-control samples. Protein concentrations were measured by the biuret reaction (12) using bovine serum albumin as the standard; by this method, 10^7 cells were found to contain 1.3 mg of protein. Intracellular P_i concentrations were calculated from a cell volume of 0.5 picoliters which was derived both from determinations of cell water (13) and from measurements of the cell diameter (9.7 μ). K_i values were determined from a Dixon plot of the reciprocal rate of P_i transport vs. inhibitor concentration.

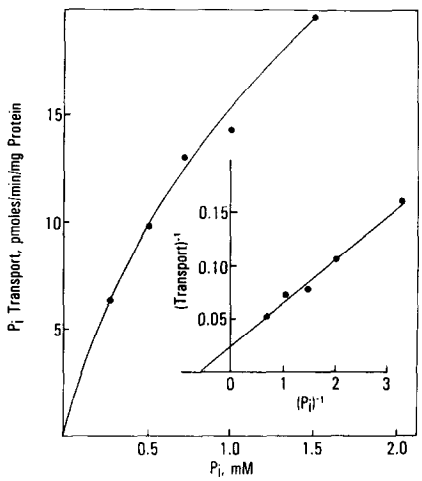

Fig. 1. Rate of P_i transport as a function of substrate concentration in the absence of added Na^+ (Buffer A). Inset, double-reciprocal plot of transport vs. P_i concentration.

Measurements of intracellular P_i. Cells (8×10^7) that had been incubated in the presence of $^{33}P_i$ (30,000 cpm/nmole) were washed twice with 0.15 M KCl to remove extracellular substrate, and then lysed by the addition (at 4°) of 0.3 M H_2SO_4 (100 µl). Aliquots of these extracts were either analyzed for protein (12) or applied directly to cellulose sheets (Kodak 6064) for chromatography (at 4°) in ethylacetate:acetic acid:H_2O (3:3:1). Developed chromatograms were dried, cut into 1.0-cm sections, and analyzed for $^{33}P_i$ (R_f = 0.45) or ATP (R_f = 0.02); the position of the latter was determined by eluting sections (1.0 cm) of a chromatogram with 1.0 ml of 0.05 M KP_i, pH 7.5, and assaying for ATP by the luciferin-luciferase procedure of Cheer et al. (14).

Results

General properties of P_i transport in the absence of Na^+. P_i transport in the absence of added Na^+ can be demonstrated in L1210 cells suspended in the same K-Hepes buffer (Buffer A) that has been employed previously to measure the uptake of MTX (4). At 1.0 mM, $^{33}P_i$ influx was linear for 20 min at 37°, and, in addition, it could be reduced by 95% upon lowering the incubation temperature to 4°. Uptake also exhibited saturation kinetics as determined from a plot of P_i influx as a function of substrate concentration (Fig. 1). From a double reciprocal plot of the data (inset, Fig. 1), a K_t value of 1.7 mM for a half-maximal rate of transport was calculated. The maximum rate of transport (V_{max}) was 50 pmoles/min/mg protein.

$^{33}P_i$ influx was inhibited by the presence of increasing amounts of *unlabeled* P_i added to the assay medium, confirming that this $^{33}P_i$ uptake component was primarily carrier mediated. Inhibition reached 80% at the

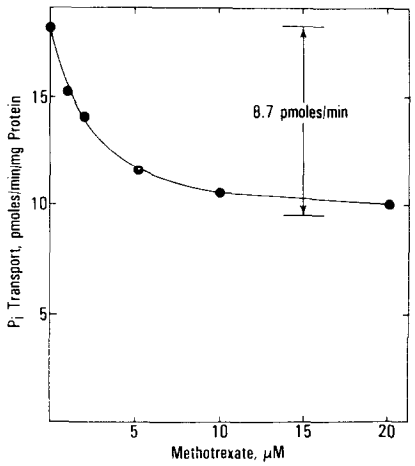

Fig. 2. Rate of P_i transport as a function of MTX concentration in the absence of added Na^+ (Buffer A). $^{33}P_i$ concentration, 1.0 mM.

highest concentration (15 mM) of unlabeled substrate tested. From a Dixon plot of the latter data, the K_i for unlabeled P_i was 2.1 mM, a value similar to the K_t of 1.7 mM calculated from the data in Fig. 1.

Evidence for P_i transport via the MTX carrier system. P_i transport in the absence of added Na^+ was also inhibited by MTX (Fig. 2). The P_i transport component affected by MTX represented approximately 50% of total uptake, and proceeded at a rate (8.7 pmoles/min/mg protein) that is in the same range as the V_{max} (15 pmoles/min/mg protein) for MTX transport. Moreover, the concentration of MTX (2 μM) required to inhibit this component by 50% approximated the K_t (1.5 μM) for transport of MTX. A comparable (43%) inhibition of P_i uptake was also observed with F-MTX (20 μM), a potent inhibitor of MTX influx which does not enter L1210 cells under the conditions employed (15).

Since the MTX transport system can be irreversibly inactivated by treatment of the cells with carbodiimide-activated MTX (16), the effect of this reagent upon P_i transport was investigated. Cells exposed to activated MTX, and then washed to remove the agent, were found to lose 50% of their capacity for Na^+-independent transport of P_i (Table 1). This parallels the result obtained by inhibiting uptake with an excess of MTX or F-MTX added directly to the assay medium (cf. Fig. 2). Residual P_i uptake was

Table 1. Effect of MTX on P_i transport (in the absence of Na^+) in control cells or in cells pretreated with activated MTX

Pretreatment with Activated MTX	Assay Addition (20 μM)	P_i Transport	Inhibition
		pmoles/min/mg protein	%
-	None	21.9	0
-	MTX	11.0	50
+	None	10.7	51
+	MTX	10.0	54

Control cells or cells pretreated for 5 min at 37° with 40 μM activated MTX (in buffer A) were washed and resuspended in buffer A prior to measurement of P_i influx in the absence or presence of 20 μM MTX.

insensitive to the subsequent addition of MTX, demonstrating that activated MTX and MTX had inhibited the same transport component.

Na^+-dependent transport of P_i in L1210 cells. The ability of the MTX carrier protein to also transport P_i (cf. Table 1 and Fig. 2) is consistent with the hypothesis that P_i is an exchange anion for MTX and that a gradient of P_i acts as an energy source for drug uptake. Since gradients of P_i are maintained in other mammalian cells by a Na^+-dependent transport system (17-20), experiments were performed to determine whether L1210 cells also have this capacity. Cells suspended in buffer containing 140 mM Na^+ (Buffer B, pH 7.4) accumulated P_i in a linear fashion for at least 30 min at 37° and at a rate that was 20-fold higher than for cells suspended in buffer in which the NaCl was replaced with an equal concentration of either KCl or choline-Cl. A double-reciprocal plot of P_i uptake as a function of P_i concentration gave an apparent K_t value of 0.42 mM, and a V_{max} of 1.1 nmoles/min/mg protein. When the Na^+ concentration was varied by replacement with K^+, the rate profile appeared sigmoidal (Hill coefficient = 1.14) and half-maximal uptake of P_i was estimated (from a double-reciprocal plot of the data) to occur at 170 mM Na^+. Sigmoidicity increased (n = 1.44) when the above experiment was performed at pH 7.8, but decreased (n = 1.04) when the pH was lowered to pH 7.0.

The ability of various anions to inhibit the Na^+-dependent transport system is shown in Table 2. From inhibition constants (K_i values) derived

Table 2. Inhibition constants of various anions for the Na^+-dependent P_i transport system

Anion	K_i
	mM
P_i	0.27
Pyrophosphate	0.39
Arsenate	2.6
Sulfate	10
AMP	>10
Methotrexate	>10

K_i values were determined in Buffer B from a Dixon plot of the reciprocal rates of the P_i transport rate vs. inhibitor concentration. $^{33}P_i$ concentration, 0.5 mM.

for these compounds, the order of effectiveness was unlabeled P_i > pyrophosphate > arsenate > sulfate > AMP and MTX. Values for K_i ranged from 0.27 mM for P_i (which is comparable to the measured K_t of 0.32 mM), to greater than 10 mM for MTX and AMP.

Status of accumulated P_i. Cells that had been exposed to $^{33}P_i$ (0.2 mM) for various times (at 37°) were analyzed by TLC for the accumulation of unmetabolized P_i (Fig. 3). After a short time interval (2 min), the radioactivity that had entered the cells remained largely as P_i (60% of the total). Upon further incubation, the proportion of P_i decreased relative to total uptake, although its intracellular concentration after 20 min

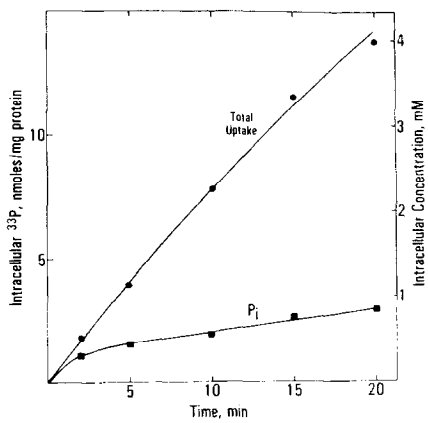

Fig. 3. Intracellular accumulation of P_i as a function of time. Cells were incubated in Buffer B containing 5 mM glucose and 0.2 mM $^{33}P_i$ for the indicated times, washed twice to remove extracellular substrate, resuspended in H_2SO_4, and analyzed for $^{33}P_i$.

was relatively high (1 mM) and represented a 5-fold increase over that in the extracellular compartment (0.2 mM).

Significant changes in the intracellular level of $^{33}P_i$ were observed upon exposure of cells (in P_i-free medium) to either azide or glucose (Table 3). After a 10-min incubation at 37°, control cells were found to contain 1.2 mM P_i, while the P_i concentration rose to 3.6 mM in cells to which azide was added during the same incubation period. Conversely, the addition of glucose lowered the P_i level to 0.50 mM after a 10-min incubation and to only 0.36 mM after 20 min at 37°. When the P_i level in the cells was expressed relative to total ^{33}P (i.e., organic plus inorganic phosphates), azide increased P_i to 80% of total ^{33}P, while only 11% of the total radioactivity remained as P_i after incubation (for 10 min) with glucose. This compares with 27% P_i in control cells.

Discussion

P_i enters L1210 cells via at least two transport systems; a low capacity, Na^+-independent route that can be inhibited by MTX (cf. Table 1 and Fig. 2), and a high capacity, MTX-insensitive system that requires Na^+. The latter system is comparable to P_i transport systems in other mammalian cells (17-20) with regard to K_t value for P_i, Na^+-dependence, and sigmoidal kinetics observed as a function of Na^+ concentration. The strict dependence on Na^+ suggests further that the L1210 system can utilize the Na^+ gradient to concentrate free P_i. Direct measurements confirmed that L1210 cells can accumulate P_i, with concentration gradients being observed in the range of 5-fold. The extent of P_i accumulation may be even higher than 5-fold, due to the dilution of accumulated $^{33}P_i$ with unlabeled P_i within the cell. Conversely, P_i levels may have been overestimated by the hydrolysis of organophosphate compounds during the extraction procedure, but this is not likely in the present study since up to 92% of total ^{33}P could be recovered as organophosphates in cells incubated with glucose (Table 3).

A relationship between P_i and MTX transport was derived from the observation that L1210 cells possess a transport route for P_i which is sensitive

Table 3. Effect of Azide and Glucose on Intracellular Levels of P_i

Addition (5mM)	Incubation Time	Intracellular $^{33}P_i$	$^{33}P_i$
	min	mM	% of Total Label
None	10	1.2	27
Azide	10	3.6	80
Glucose	10	0.50	11
Glucose	20	0.36	8

Cells (in Buffer B) were preincubated for 20 min at 37° with 0.2 mM $^{33}P_i$ (to label the P_i pool), washed to remove extracellular $^{33}P_i$, and then incubated (at 37°) for an additional 10 or 20 min with either no addition, 5 mM azide, or 5 mM glucose. Intracellular levels of $^{33}P_i$ were then determined by thin-layer chromatography as described in Materials and Methods.

to MTX (cf. Table 1 and Fig. 2). This P_i uptake component was identified as the MTX transport system since it: (A) was inhibited by 50% at a concentration of MTX (2 μM) that is comparable to the K_t (1.5 μM) for MTX influx; (B) could be blocked by either pretreatment with an irreversible-labeling agent (carbodiimide-activated MTX) (cf. Table 1) or by an inhibitor of MTX influx (F-MTX) that is not readily transported across the cell membrane (15); and (C) proceeded at a rate (8.7 pmoles/min/mg protein) that is in the same range as the V_{max} (15 pmoles/min/mg protein) for MTX transport.

The significance of a P_i-transporting capacity for the MTX carrier system is that it allows intracellular P_i to act as an exchange anion for the uptake of extracellular MTX. Accumulated P_i (taken up via a separate Na^+-dependent route) can then serve as an energy source for the concentrative uptake of the drug. A coupling mechanism of this type also provides an explanation for the effect of cellular metabolism on MTX uptake. It has been shown that the ability of cells to accumulate MTX increases in cells exposed to metabolic inhibitors (e.g., azide or iodoacetate) and likewise decreases in the presence of energy sources (e.g., pyruvate and glucose) (4,5,9,10). These effects, which have been related by others to an ATP-dependent efflux mechanism (5), may instead be a direct result of fluctuations in the intracellular level of P_i. Consistent with the latter suggestion, the present study shows that cells exposed to azide do have elevated levels

of P_i (Table 3), and thus would have an increased gradient of P_i (relative to untreated cells) for driving MTX uptake. Similarly, glucose could have a reductive effect on steady-state levels of MTX by its ability (cf. Table 3) to decrease the intracellular pool of P_i.

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