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Properties of an Anion/H^+ Cotransport System in L1210 Cells that Utilizes Phthalate as a Nonphysiological Substrate

Gary B. Henderson and Edward M. Zevely
Division of Biochemistry, Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California 92037

Summary. [^{14}C]Phthalate is transported into L1210 cells via two separate routes, an anion exchange system whose primary substrates are folate compounds, and a second less active system which is sensitive to bromosulfophthalein. When the principal uptake component was blocked by a specific irreversible inhibitor of this system, the remaining route (at pH 7.4) appeared to be saturable and was inhibited by several anions in addition to bromosulfophthalein (K_i = 2 μM), including 8-anilino-1-naphthalein sulfonate (K_i = 25 μM), unlabeled phthalate (K_i = 500 μM), and chloride (K_i = 3500 μM). A pronounced effect by pH was also observed. Influx and total uptake of phthalate both increased progressively with decreasing pH and reached values that were 20-fold higher at pH 6.0, compared with pH 7.4. This pH-dependent increase could be blocked, however, by the addition of compounds (nigericin and carbonylcyanide *m*-chlorophenylhydrazone) which, in combination, collapse proton gradients. Phthalate efflux was relatively insensitive to changes in extracellular pH but could be inhibited (up to 90%) by bromosulfophthalein. Several other anions also inhibited efflux, but to a lesser extent, while chloride, phthalate, lactate, glycolate and acetate enhanced efflux up to 1.8-fold. Efflux also increased at pH 6.0, but not at pH 7.5, upon addition of nigericin and carbonylcyanide *m*-chlorophenylhydrazone. These results suggest that phthalate is a nonphysiological substrate for a carrier system which mediates transport via an anion/H^+ symport mechanism. This system is not the lactate/H^+ symport carrier of L1210 cells since: (A) phthalate and lactate influx were inhibited to differing degrees by various anions; and (B) lactic anhydride inhibited the influx and efflux of lactate but had no effect on the transmembrane movement of phthalate. The specificity of this system suggests that its primary anion substrate may be chloride.

Key Words phthalate · H^+/anion cotransport · H^+/chloride cotransport · H^+/lactate cotransport · intracellular pH control · bromosulfophthalein

Introduction

Transport systems have been identified that mediate the electroneutral movement of anions across plasma membranes. Electroneutrality is desirable since this counteracts the outward driving force that the membrane potential exerts during the transmembrane movement of anions. The two most common mechanisms for achieving electroneutrality involve either the exchange of intracellular and extracellular anions of equal charge or the cotransport of anions with cations. A widely studied example of an anion exchange mechanism is the chloride/bicarbonate exchange system that is present in erythrocytes [1, 11, 15] and other cells [8, 13, 14]. The carrier that mediates this process (band 3) can accommodate a broad spectrum of small, monovalent anions, although chloride and bicarbonate are transported at a much higher V_{max} than other anion substrates. Specificity is thus achieved by variations in the maximum transport rate of specific substrates. A broad substrate specificity is also associated with the system that mediates the exchange uptake of methotrexate and other folate compounds in L1210 cells [6, 8–10]. In this case, transport appears to occur via a divalent anion exchange and specificity is achieved by varying the binding affinity for substrates. Maximum rates of transport are approximately the same for folate compounds, phosphate, sulfate, AMP, and other anion substrates [7–9]. The basis for a broad specificity appears to be that the carrier protein accommodates both folate compounds and structurally diverse exchange anions at a single binding site.

The broad substrate specificity of the folate transport system of L1210 cells was first suggested from the ability of various anions to mediate the transtimulation of [^{3}H]methotrexate efflux [9] and was subsequently verified by the ability of this system to mediate the direct uptake of predicted anion substrates including inorganic phosphate [7], sulfate [8], and *o*-phthalate [10]. Transport studies with *o*-phthalate showed further that the influx of this compound occurs with a relatively high efficiency and that the folate transport system is the primary route

for *o*-phthalate uptake. An additional finding in the latter study was that a second uptake route is also available for *o*-phthalate. In the present study, the properties of this second component have been determined. The results indicate that uptake via this route is carried-mediated and that a phthalate/H^+ symport mechanism is employed to move the anionic substrate into the cell. Specificity measurements indicate further that the primary anion substrate for the transport system may be chloride.

Materials and Methods

Chemicals

o-[7-^{14}C]Phthalic acid (5 mCi/mmol) and [U-^{14}C]L-lactic acid (105 mCi/mmol) were obtained from ICN. Radiochemical purity of the [^{14}C]phthalate was 99% by thin-layer chromatography on cellulose sheets using 20 mM MES[1]–200 mM NaCl, pH 6.0, as the solvent. NHS-methotrexate was prepared as described previously [5, 9] by dissolving methotrexate (2.2 mg), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (7.8 mg), and N-hydroxysuccinimide (5.8 mg) in 2.0 ml anhydrous dimethylsulfoxide and allowing the mixture to stand for 60 min at 23°C. The concentration of NHS-methotrexate was determined from the extinction coefficient for methotrexate of 18.9 at 302 nm and pH 7. Lactic anhydride was prepared each day just prior to use by the procedure of Johnson et al. [12]. Lactic acid (9.0 mg) was dried over phosphorous pentoxide for 16 hr, dissolved in 1.0 ml of anhydrous tetrahydrofuran, and cooled to −15°C. N-Methylmorpholine (44 μl) and isobutylchloroformate (38 μl) were then added and the mixture incubated for 5 min at −15°C. After warming to 4°C, the sample was centrifuged at 10,000 × *g* (5 min, 4°C). The supernatant solution was recovered, evaporated under vacuum to an oil, dissolved in 1.0 ml anhydrous dimethylsulfoxide and used immediately. All other chemicals were of the highest purity available commercially and were used without further purification.

Cells

L1210 mouse leukemia cells were grown as described previously [5], washed with the desired buffer, and resuspended to a density of 3 × 10^7 cells/ml. Buffers employed as suspending media for cells were as follows: HBS (HEPES-buffered saline), 20 mM HEPES-140 mM NaCl-5 mM KCl-1 mM $MgCl_2$, pH 7.4 with NaOH; MHS (Mg-HEPES-sucrose), 20 mM HEPES-225 mM sucrose, pH 7.4 with MgO; MBS (MES-buffered saline), 20 mM MES-140 mM NaCl-5 mM KCl-1 mM $MgCl_2$, pH 6.0 with NaOH; and MMS (Mg-MES-sucrose), 20 mM MES-225 mM sucrose, pH 6.0 with MgO.

[1] *Abbreviations:* MES, 2-(N-morpholino)ethanesulfonate; HEPES, 4-2(2-hydroxyethyl)-1-piperazine-ethanesulfonate; DMO, 5,5-dimethyloxazolidine-2,4-dione; HBS, HEPES-buffered saline; MBS, MES-buffered saline; MHS, Mg-HEPES-sucrose buffer; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; NHS-methotrexate, N-hydroxysuccinimide ester of methotrexate.

Treatment with NHS-Methotrexate

Treatment with NHS-methotrexate was accomplished by exposing freshly harvested cells (in either HBS or MHS, pH 7.4) to 10 μM NHS-methotrexate for 5 min at 23°C. The cells were then centrifuged, washed with 25 ml of the desired buffer, and resuspended to 3 × 10^7/ml in the same buffer.

Influx Measurements

Phthalate influx was determined in assay mixtures containing untreated or NHS-methotrexate-treated cells (3 × 10^7), the desired additions, and 20 nmol of [^{14}C]phthalate in 1.0 ml of buffer. After incubation at 37°C for 5 min, the cells were chilled to 4°C, collected by centrifugation at 1000 × *g* (5 min, 4°C), washed with 4 ml of ice-cold 0.15 M NaCl, resuspended in 0.5 ml saline, and analyzed for radioactivity. Uptake at 0°C served as the control. Influx is reported in picomoles of phthalate transported/min/mg protein. Protein concentrations were determined from the Biuret reaction [4] using bovine serum albumin as the standard. Intracellular concentrations of phthalate were calculated on the basis of a cell volume of 5 × 10^{-10} ml [2].

Lactate influx was measured by combining cells (2 × 10^7) and the desired additions in 0.95 ml of MBS or MMS buffer, pH 6.0, and preincubating the mixtures at 37°C for 2 min. [^{14}C]Lactate (50 μl, 1.0 mM) was then added and the incubation was continued at 37°C. After 30 sec, the cells were diluted with 7 ml of ice-cold 0.15 M NaCl, recovered by centrifugation at 4°C (3 min, 1000 × *g*) washed with 4 ml of saline, and analyzed for radioactivity. Uptake at 0°C served as a control. Influx was reported in nanomoles of lactate transported/min/mg protein.

Efflux Measurements

Cell loading with phthalate was achieved by combining cells (6 × 10^8) and [^{14}C]phthalate (400 nmol) in 20 ml of the MHS, pH 7.4, and incubating the mixture for 15 min at 37°C. The cells were then recovered by centrifugation at 1000 × *g* (5 min, 4°C), and suspended in buffer containing 10 μM NHS-methotrexate. After 5 min at 23°C, the cells were centrifuged and resuspended in buffer to 3 × 10^7/ml. The desired compounds were then added, and the cells were incubated for various times at 37°C, diluted with 7 ml of ice-cold saline, collected by centrifugation, and analyzed for associated radioactivity. Loading with lactate was achieved similarly except that assay mixtures contained cells (4 × 10^8) and [^{14}C]lactate (1 μmol) in 20 ml of MBS, pH 6.0 and the mixtures were incubated for 5 min at 37°C.

Determinations of Intracellular pH

Intracellular pH was determined [16] by measuring the equilibrium distribution of the weak acid, 5,5′-dimethyl [2-^{14}C]oxazolidine-2,4-dione (DMO), across the cell membrane. Cells (4 × 10^7) were suspended in 1.0 ml of buffer containing the desired additions and 2.0 μM [^{14}C]DMO, incubated at 37°C for a sufficient time (1 min) to achieve a steady state, collected by centrifugation and analyzed for accumulated radioactivity. Uptake of [^{14}C]phthalate at 0°C (conditions under which internalization of this anion is minimal) served as a control for extracellular water space. Calculations of intracellular pH employed a cell volume of 5 × 10^{-10} ml [2].

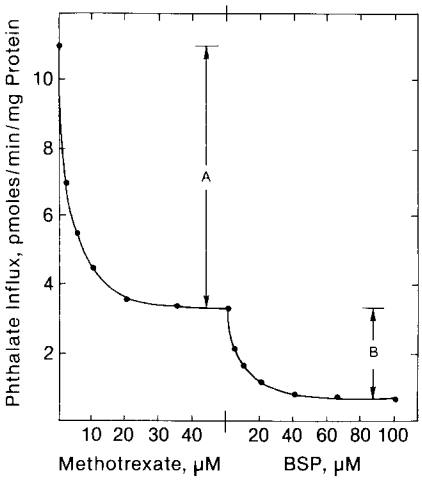

Fig. 1. Inhibition of phthalate influx by increasing concentrations of methotrexate and bromosulfophthalein. Influx of phthalate in the presence of the indicated inhibitors was determined as described in Materials and Methods. Component A, portion of phthalate influx sensitive to methotrexate; component B, portion of phthalate influx sensitive to methotrexate plus bromosulfophthalein (BSP)

Table 1. Inhibition of [^{14}C]phthalate influx by various anions[a]

Anion	Inhibition by 50% (mM)
Phthalate	0.5
Benzoate	1.2
Anthranylate	1.0
Pyrrole-2-carboxylate	1.6
Acetate	15
L-Lactate	10
Glycolate	4.3
Allantoate	7.5
Chloride	3.5
Phosphate	3.0
BSP	0.002
ANS	0.025
Cholate	0.13
Methotrexate	1.2
NAP-taurine	0.55

[a] Inhibition by the Na^+ salts of the indicated anions was measured on the bromosulfophthalein-sensitive portion of phthalate influx in MHS, pH 7.4. BSP, bromosulfophthalein; ANS, 8-anilino-1-napththalein sulfonate; NAP-taurine, N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate.

Results

Separation of Phthalate Influx Components

Phthalate influx in L1210 cells has been shown to proceed via at least two components [10]. The major route is the methotrexate influx carrier as judged from its sensitivity to reversible inhibition by various anions and to irreversible inhibition by an N-hydroxysuccinimide ester of methotrexate (NHS-methotrexate). The second route, which is activated in buffers lacking chloride ions [10], was identified by its sensitivity to bromosulfophthalein. Separation of phthalate influx into these individual components is shown in Fig. 1. In a buffered sucrose medium (MHS) at pH 7.4, the portion of phthalate influx that proceeded via the methotrexate influx carrier (component A, Fig. 1) was 70%. This value was derived from the maximum inhibition achieved at high concentrations of methotrexate. Subsequent additions of increasing amounts of bromosulfophthalein (Fig. 1) revealed the second component of phthalate influx (component B), which comprised 24% of the total. The remaining influx (6% of the total) was not sensitive to either inhibitor at the concentrations tested. The component of phthalate influx that could be inhibited by methotrexate was also eliminated by exposure to NHS-methotrexate. Hence, an analysis of phthalate transport via the bromosulfophthalein-sensitive route (component B), without interference from the methotrexate carrier system (component A), can be accomplished conveniently and efficiently by first treating the cells with NHS-methotrexate.

Kinetics of Phthalate Influx

The dependence of phthalate influx on substrate concentration was measured to determine whether the bromosulfophthalein-sensitive influx component followed saturation kinetics. Influx (in cells treated with NHS-methotrexate) was found to be linear at phthalate concentrations between 20 and 100 μM and then to gradually decrease thereafter up to a concentration of 250 μM. Saturation of influx was thus implicated but could not be clearly established under these experimental conditions. Influx at 20 μM [^{14}C]phthalate, however, could be inhibited to a maximum of 90% by unlabeled phthalate, indicating that a carrier-mediated process was operative. The K_i for phthalate was 500 μM, a value consistent with the low saturability of this system. Assuming that the K_t for [^{14}C]phthalate was also 500 μM, the V_{max} for influx in MHS, pH 7.4, was estimated to be 70 pmol/min/mg protein.

Specificity of the Bromosulfophthalein-Sensitive Component

Phthalate influx in cells treated with NHS-methotrexate is inhibited by a variety of anions (Table 1). The most effective inhibitors included bromosulfo-

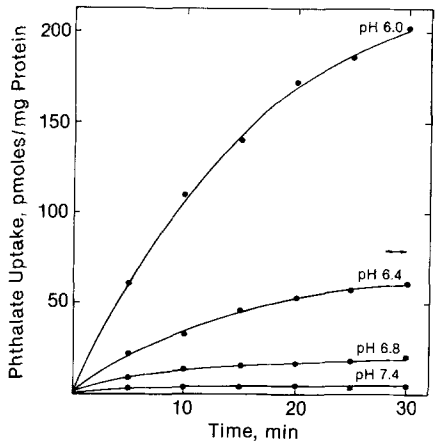

Fig. 2. Effect of pH on the time-dependent uptake of phthalate. Cells were suspended in buffer consisting of (mM): 10 MES, 10 HEPES, 140 NaCl, 5 KCl and 1 $MgCl_2$, adjusted to the indicated pH by the addition of NaOH, and examined for phthalate uptake with time. The arrow corresponds to the intracellular phthalate concentration at which equilibration of the substrate across the membrane occurs

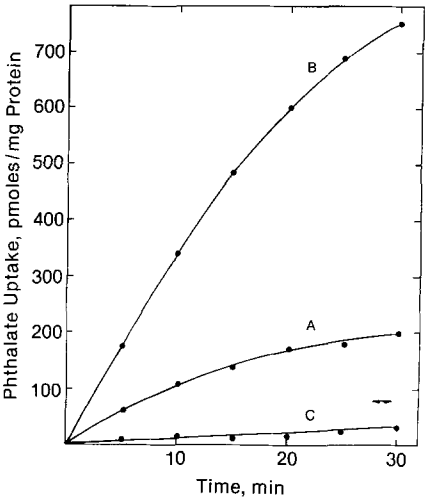

Fig. 3. Effect of buffer composition and proton-conducting ionophores on the uptake of phthalate at pH 6.0. Uptake was measured in: *A*, MBS (MES-buffered saline), pH 6.0; *B*, MMS (Mg-MES-sucrose), pH 6.0; or *C*, MBS, pH 6.0, containing 2 μg/ml each of nigericin and CCCP. The arrow corresponds to the intracellular phthalate concentration at which equilibration of the substrate across the membrane occurs

phthalein, 8-anilino-1-naphthalene sulfonate, cholate and phthalate. Inhibition was also observed with chloride, whose K_i of 3.5 mM was substantially lower than internal or external concentrations of this anion usually present under physiological conditions.

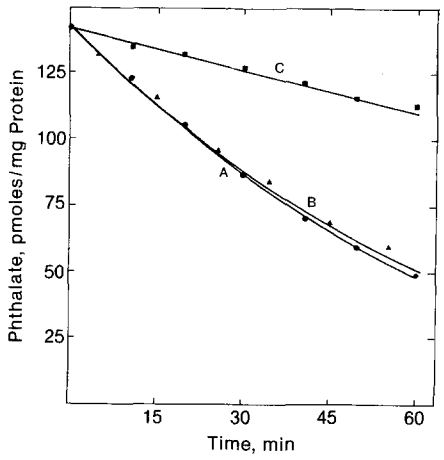

Fig. 4. Separation of efflux routes for phthalate by specific inhibitors. Efflux was measured in: *A*, untreated cells; *B*, cells pretreated with 10 μM NHS-methotrexate; and *C*, untreated cells in the presence of 50 μM bromosulfophthalein. Load and efflux buffer, MHS, pH 7.4

Effect of pH on Phthalate Uptake

Phthalate uptake via the bromosulfophthalein-sensitive route was highly sensitive to the pH of the external medium. In a buffered saline medium, phthalate influx and total uptake were both found to increase progressively as the pH was reduced from 7.4 to 6.0 (Fig. 2). The extent of this increase was substantial since the difference in uptake between 6.0 and 7.4 exceeded 20-fold. Total uptake at pH 6.0 was also concentrative, with intracellular levels of phthalate exceeding extracellular levels by greater than twofold after 30 min (*see* Fig. 2).

Phthalate uptake at pH 6.0 could be increased further by transferring the cells from a saline buffer (MBS) to a buffered sucrose medium (MMS). Influx increased an additional 2.6-fold and uptake reached a concentration gradient in excess of 9-fold after 30 min. The sensitivity of influx to bromosulfophthalein (K_i = 25 μM) remained high under these conditions, with maximum inhibition exceeding 90%. Unlabeled phthalate inhibited [^{14}C]phthalate influx in MMS buffer with a K_i of 1.0 mM, which is slightly higher than the K_i of 0.5 mM observed in MHS, pH 7.4 (*see* Table 1). From the influx rate at 20 μM [^{14}C]phthalate and a K_i of unlabeled phthalate of 1.0 mM, the V_{max} for phthalate influx in MMS, pH 6.0, was calculated to be 2000 pmol/min/mg protein, a value 30-fold higher than observed in HBS, pH 7.4. In MBS, pH 6.0, K_i values for bromosulfophthalein (120 μM) and for unlabeled phthalate (5 mM) each increased fivefold and the calculated V_{max} decreased

Table 2. Effect of various anions on [^{14}C]phthalate efflux[a]

Anion	Concentration (mM)	Initial efflux (pmol/min/mg)	Response (% of control)
None	—	1.85	0
Phthalate	5	2.09	+13
Benzoate	5	1.02	−45
Anthranylate	5	0.59	−68
Pyrrole-2-carboxylate	5	1.50	−19
Acetate	10	2.70	+46
L-Lactate	10	3.05	+65
Glycolate	10	3.20	+73
Allantoate	10	1.52	−18
Chloride	20	2.63	+42
Phosphate	20	1.89	+ 2
BSP	0.2	0.43	−77
ANS	0.2	0.98	−47
Cholate	0.5	1.04	−44
Methotrexate	5.0	1.70	− 8
NAP-taurine	2.0	1.50	−19

[a] Cells were loaded to 130 pmol/mg protein. Load and efflux buffer, MHS, pH 7.4. BSP, bromosulfophthalein; ANS, 8-anilino-1-naphthalein sulfonate; NAP, taurine, N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate.

twofold, relative to corresponding values obtained in buffered sucrose medium at the same pH. When a combination of nigericin and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was added to cells in MBS, pH 6.0, substantial inhibition of both influx and total uptake was observed (Fig. 3). Conversely, these same inhibitors had no effect on phthalate uptake in HBS, pH 7.4 (*data not shown*). The pH gradient across the membrane of L1210 cells at pH 6.0 is collapsed by the addition of nigericin and CCCP. Cells suspended in MBS, pH 6.0, exhibited an initial intracellular pH of 7.3, which then decreased slowly to 6.6 after 20 min at 37°C. The internal pH of cells in the same pH 6.0 buffer but containing nigericin and CCCP was 6.1 and it did not change over a 20-min incubation interval at 37°C. Prior treatment of the cells (5 min, 23°C) with 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) in an amount (20 μM) sufficient to block the general anion transport system of L1210 cells [8] also had no effect on phthalate influx, either at pH 6.0 or at pH 7.4.

Phthalate Efflux

The efflux of phthalate proceeds via a principal component which can be inhibited by bromosulfophthalein and a second inhibitor-insensitive route [10]. No detectable efflux of phthalate occurs via the methotrexate exchange carrier. The different components of phthalate efflux are illustrated by the results shown in Fig. 4. Efflux proceeded with an initial rate of 2.3 pmol/min/mg protein in control cells (curve A) and was not affected (curve B) by prior treatment with NHS-methotrexate, although substantial inhibition could be achieved upon addition of bromosulfophthalein (curve C). The extent of inhibition ranged from 75 to 80% (*see* Fig. 4) and was half-maximal at a bromosulfophthalein concentration of 5 μM [10]. When the various anions that had been tested as inhibitors of phthalate influx (Table 1) were examined for an effect on phthalate efflux, some anions were found to enhance efflux, while others were inhibitory (Table 2). Inhibition was generally associated with anions containing bulky and/or aromatic substituents (e.g., 8-anilino-1-naphthalene sulfonate, cholate, anthranylate and benzoate), while stimulation occurred with several small monovalent anions (e.g., chloride, acetate, lactate and glycolate). Stimulation by the latter anions could be blocked by bromosulfophthalein but was not affected by prior treatment of the cells (5 min, 23°C) with 20 μM DIDS. At pH 6.0, phthalate efflux proceeded at a slightly higher initial rate than at pH 7.4 (Table 3) and was more sensitive to bromosulfophthalein (90% inhibition at 200 μM). The combination of nigericin plus CCCP increased initial efflux by 14-fold at pH 6.0, but had no effect at pH 7.4 (Table 3).

Table 3. Comparative effect of nigericin plus CCCP on phthalate efflux at pH 7.4 and pH 6.0[a]

Addition	pH	Initial efflux (pmol/min/mg protein)	Stimulation by nigericin + CCCP (-fold)
None	7.4	2.2	—
Nigericin + CCCP	7.4	2.2	1.0
None	6.0	2.7	—
Nigericin + CCCP	6.0	37	14

[a] Cells were loaded to 110 pmol/mg protein in MHS, pH 7.4, treated with NHS-methotrexate, resuspended in HBS, pH 7.4, or MBS, pH 6.0, and then analyzed for initial efflux in the absence and presence of 2 μg/ml each of nigericin and CCCP. Stock solutions of nigericin and CCCP were prepared in dimethylsulfoxide; final concentration of dimethylsulfoxide in assay mixtures, 0.5%.

Table 4. Comparative inhibition of phthalate and lactate influx by various anions[a]

Anion	Inhibition by 50%	
	Phthalate influx (μM)	Lactate influx (μM)
BSP	120	110
NAP-taurine	3000	600
α-Cyano-4-hydroxycinnimate	3000	120
Probenecid	200	700
Quercetin	2	10

[a] Substrate concentrations: [^{14}C]phthalate, 20 μM; [^{14}C]lactate, 50 μM. Assay buffer, MBS, pH 6.0; BSP, bromosulfophthalein; NAP-taurine, N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate.

Characteristics of Lactate/H^+ Transport in L1210 Cells

The observation that phthalate uptake is enhanced by lowering the pH and that efflux is stimulated by proton-conducting ionophores (nigericin plus CCCP) is consistent with phthalate transport occurring via a phthalate/H^+ symport mechanism. Since a similar mechanism has been proposed for the transport of lactate [3, 12, 18], phthalate and lactate influx were compared in an effort to determine whether phthalate was being transported via the lactate carrier system. A plot (*not shown*) of the time dependence of lactate accumulation by cells in MBS, pH 6.0, revealed that uptake was linear for approximately 30 sec and reached a steady state after 5 min at 37°C. Initial influx of lactate was 1.8 to 2.5 nmol/min/mg protein, which corresponded to a rate that was 200-fold greater than for phthalate. Transfer of the cells to a buffer lacking chloride ions (MMS, pH 6.0), had no effect on the influx of lactate, unlike phthalate whose influx under these same conditions increased 2.6-fold (*see* Fig. 3). Various anions also differentially inhibited the influx of lactate and phthalate (Table 4). NAP-taurine and α-cyano-4-hydroxycinnimate were much more effective inhibitors of lactate influx than phthalate influx, while probenecid and quercetin inhibited phthalate influx more effectively than lactate influx. An exception was bromosulfophthalein, which inhibited both processes with approximately the same K_i value. A different response was also observed with lactic anhydride, a covalent inhibitor of the lactate carrier system [12]. This compound inhibited lactate influx half-maximally at a concentration of 40 μM but had little effect on the influx of phthalate at concentrations up to 250 μM (Fig. 5).

The efflux of lactate could also be inhibited by lactate anhydride (Table 5). The extent of inhibition was 93% in cells loaded with [^{14}C]lactate, treated with 200 μM lactic anhydride, and analyzed for initial efflux. In contrast, lactic anhydride treatment had no effect on the efflux of [^{14}C]phthalate (Table 5).

Discussion

Phthalate can be transported into L1210 mouse cells via at least two components [10]. The primary route is the same system which mediates the uptake of methotrexate and other folate compounds. This conclusion was supported by a parallel sensitivity of both methotrexate and phthalate influx to competitive inhibition by various anions, irreversible inhibition of both processes by NHS-methotrexate, and similar fluctuations in influx with the ionic composition of the medium. Utilization of the folate carrier system for the transport of phthalate had been predicted by prior studies on the structural requirements of anion substrates for this system [9]. In addition, phthalate is bound by this system with a relatively high affinity, as is the case with other divalent anions with aromatic substituents [9]. The second influx route for phthalate, which accounted for the major portion of the remaining uptake, was identified by its high sensitivity to bromosulfophthalein (K_i = 5 μM). An unusual property of this system is that it is inhibited in saline buffers.

The bromosulfophthalein-sensitive portion of phthalate influx has been characterized in the present study and the findings indicate that trans-

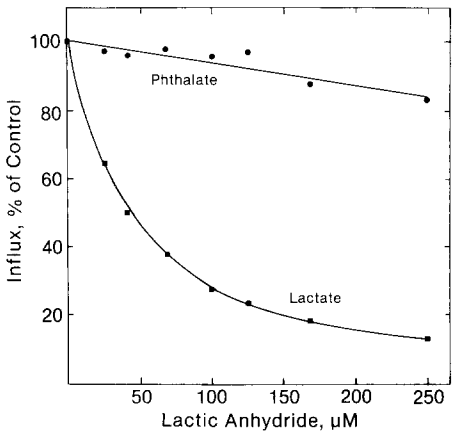

Fig. 5. Comparative inhibition of phthalate and lactate influx by increasing concentrations of lactic anhydride. Cells were pretreated with the indicated concentrations of lactic anhydride for 60 min at 0°C, washed, and then analyzed for phthalate or lactate influx as described in Materials and Methods. Pretreatment and assay buffer, MBS, pH 6.0. Initial influx: phthalate, 15 pmol/min/mg protein; lactate, 1900 pmol/min/mg protein

Table 5. Comparative effect of lactic anhydride treatment on lactate and phthalate efflux at pH 7.4[a]

Addition	Lactate efflux (pmol/min/mg protein)	Phthalate efflux
None	1500	2.5
Lactic anhydride	110	2.5

[a] Cells were loaded with [^{14}C]lactate or [^{14}C]phthalate (*see* Materials and Methods) treated with 200 μM lactic anhydride for 30 min at 0°C, washed with 200 ml of HBS, pH 7.4, suspended in the same buffer containing 2 μg/ml each of nigericin and CCCP, and analyzed for initial efflux. Cell load: [^{14}C]lactate, 1480 pmol/mg protein; [^{14}C]phthalate, 137 pmol/mg protein.

port via this route is mediated by a membrane-associated carrier protein. Inhibition was observed with a wide range of anions, in addition to bromosulfophthalein, and in some cases binding was nearly complete and occurred with a relatively high affinity (*cf.* Tables 1 and 4). Sensitivity to anions also extended over a range of pH values (7.4 to 6.0), and evidence for saturation kinetics was obtained from the ability to inhibit [^{14}C]phthalate influx with unlabeled substrate.

The effect of pH on the movement of phthalate across the membrane provided insight into possible mechanisms of transport. At pH 6.0, both influx and total uptake of phthalate were 20-fold higher than at pH 7.4. This increase in uptake was not due to an increase in binding affinity for phthalate since unlabeled phthalate was a less effective transport inhibitor (by twofold) at the lower pH. Similarly, passive diffusion did not appear to increase significantly under these conditions since a major portion of influx (90%) remained sensitive to anions, including bromosulfophthalein, quercetin, and unlabeled phthalate (*see* Table 4). Ionophores (nigericin and CCCP) which, in combination, collapse proton gradients also had pronounced effects on phthalate transport, but only at low pH. These compounds inhibited influx and stimulated efflux at pH 6.0, but had no effect on either process at pH 7.4. The proposed explanation for these pH and ionophore effects is that a carrier protein mediates phthalate transport and it functions via an anion/H^+ symport mechanism. In a mechanism of this type, reorientation of the binding site(s) can occur only when both or neither substrate is bound to the carrier protein, while reorientation is prohibited when the carrier contains only one bound substrate. The consequence of decreasing the pH would then be to increase the availability of protons for binding to the carrier protein. This in turn would enhance the proportion of transport-competent phthalate/H^+ complexes and increase influx. The proton gradient generated by a reduction in extracellular pH would be the driving force for the observed concentrative uptake of phthalate at pH 6.0 (*cf.* Figs. 2 and 3), and reduced uptake at pH 6.0 in the presence of ionophores would be a direct result of a collapse of the proton gradient across the membrane. Enhanced efflux by ionophores would also be expected at pH 6.0 since this would result in a decrease in the intracellular pH. A similar H^+-symport mechanism has been proposed for the transport of lactate [3, 18]. Phthalate transport, however, does not occur via the lactate/H^+ symporter of L1210 cells since: (A) lactate and phthalate influx are inhibited to differing degrees by various anions (Table 4); and (B) lactic anhydride, a relatively specific irreversible inhibitor of bi-directional lactate transport [12], has no effect on either the influx (Fig. 5) or efflux (Table 5) of phthalate. Transport of phthalate via the general anion carrier system was eliminated by the inability of DIDS, a specific covalent inhibitor of this system [1, 8, 11, 15], to inhibit phthalate influx.

The bromosulfophthalein-sensitive transport system which accommodates phthalate as a nonphysiological substrate may function under physiological conditions to mediate the cotransport of protons with chloride ions. One observation that supports this possibility is the high binding affinity of this system for chloride (Table 1). The K_i for chloride is 3.5 mM, which is much lower than the

concentration of chloride ions usually present outside cells in their physiological environment, or within the cell cytoplasm. As a consequence, saturation of the transport protein by chloride could be expected regardless of the orientation of the binding site. Transport activity would then depend only upon the availability of protons. Other anions such as phosphate, acetate, or lactate would be less likely to occupy the anion-binding site, due either to lower available concentrations of these anions and/or to lower binding affinities for the transport protein (*see* Table 1). Chloride appears to be an anion substrate for this transport system since it has the ability (along with certain other anions) to facilitate the countertransport of phthalate out of cells (Table 2). Chloride does not promote phthalate efflux indirectly by entering the cells via the chloride/bicarbonate exchange carrier since pretreatment with DIDS does not affect the ability of chloride to promote the efflux of phthalate.

A chloride/H^+ symport system could serve a vital role in eliminating HCl which might otherwise accumulate within cells due to the production of metabolic carbon dioxide. Carbon dioxide produced during the tricarboxylic acid cycle rapidly hydrates within cells to produce a proton and bicarbonate. The latter anion then exits the cell in exchange for chloride via the well-characterized bicarbonate/chloride exchange system and thereby causes the generation of intracellular HCl. A direct and relatively efficient route for releasing accumulated HCl might then be a chloride/H^+ cotransport system. The present results suggest that a system of this type may exist in L1210 cells and that its activity can be monitored using phthalate as a model anion substrate. Direct detection of this transport system may have been hampered previously by the pH employed for transport measurements and/or by interference from other transport systems which mediate the rapid movement of protons (e.g., the Na^+/H^+ exchange carrier) or chloride (e.g., the chloride/bicarbonate exchange carrier) across the plasma membrane. The present proposal for the expulsion of HCl via a single cotransport system also provides an alternative mechanism from that of Russell and Boron [17] who proposed that HCl may exit cells via a two-step process involving ATP hydrolysis.

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