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Substrate and Inhibitor Specificity of the Lactate Carrier of Human Neutrophils

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Summary. The substrate and inhibitor specificity of the lactic acid (Lac) transport system of human neutrophils was investigated. The ability of a variety of compounds to inhibit the influx of [^{14}C]lactate, presumably reflecting competition by substrate analogues for binding at the external translocation site, was taken as an index of affinity for the Lac carrier. pH-stat techniques were utilized to assess transportability. Results indicate a relatively low order of selectivity, the neutrophil H^+ + lactate$^-$ cotransport system demonstrating a broad acceptance of short-chain unsubstituted and substituted alkyl monocarboxylates as well as aromatic monocarboxylates. There was a slight preference for oxo, Cl, and OH substituents over other groups at the two-position of short chain alkyl fatty acids: all were readily transported across the plasma membrane at rates approaching that of L-lactate itself. Aromatic acids were not transported inward by the carrier although these compounds did permeate via simple nonionic diffusion. The neutrophil Lac carrier can be blocked by a number of cyanocinnamate derivatives, the classical inhibitors of monocarboxylate transport in mitochondria, and by dithiol compounds and sulfhydryl-reactive agents. This constellation of biochemical properties is similar to the features that characterize other well described H^+ + lactate$^-$ cotransport systems in red blood cells, Ehrlich ascites tumor cells, hepatocytes, and cardiac sarcolemmal vesicles, although significant differences exist when comparisons are made to the Na^+-dependent lactate transporter of the kidney proximal tubule.

Key Words lactic acid · monocarboxylates · membrane carriers · ion transport · neutrophil · leukocyte

Introduction

Recently, we reported on the principal pathway by which lactic acid crosses the plasma membrane of human neutrophils [32]. As these cells are absolutely dependent on anaerobic glycolysis for the generation of ATP to provide the requisite energy to drive a variety of biochemical reactions, an efficient mechanism for the disposal of lactic acid, the exclusive metabolic end-product, is of some importance. In fact, steady-state neutrophils secrete copious quantities of lactic acid into the bathing medium (~5 meq/liter of cell water · min), the flux being three- to five-fold greater than those for Na^+, K^+, or Cl^- [28, 31]. Nonionic diffusion of the neutral lactic acid species seems to play little or no role [32]. Rather, neutrophils possess a specialized transport system that bears many features in common with other monocarboxylate carriers in erythrocytes, lymphocytes, epithelia, muscle and Ehrlich ascites tumor cells [1, 2, 6, 8, 11–13, 21, 24, 34, 35, 40]. Thus, [^{14}C]lactate fluxes in neutrophils are sensitive to the organomercurial mersalyl and to UK-5099, a derivative of 2-cyano-4-hydroxycinnamate (CHC), the classical inhibitor of monocarboxylate transport in mitochondria [15, 17, 18]. Again, similar to the ionic translocation mechanism in erythrocytes [8, 9], the neutrophil Lac carrier mediates H^+ + lactate$^-$ cotransport (or some formal equivalent) since H^+ movements in association with lactate fluxes can be detected using pH-stat techniques [32].

The goal of elucidating the functional properties of the Lac carrier stems primarily from our interest in intracellular pH (pH_i) regulation in these cells. From the work of several laboratories including our own, it is readily apparent that Na^+/H^+ exchange and Cl^-/HCO_3^- exchange, while making vital contributions to the control of steady-state pH_i [14, 25, 30, 33], are not sufficient in and of themselves to set the resting pH_i level. From the evidence at hand, it would seem that the existence of an as yet unidentified Na^+-independent mechanism(s) must be invoked as playing a major role in the recovery from intracellular acidification. In a previous article [33] in which we examined the factors relating to steady-state pH_i regulation, we postulated that net proton efflux, as for example through H^+ + Cl^- cotransport (i.e., HCl secretion) via the Lac carrier may be chiefly responsible for maintaining resting pH_i at levels considerably more alkaline than expected on thermodynamic grounds for passive H^+ distribution. With the long-range aim of providing definitive proof

for this scheme, we felt it appropriate to characterize the substrate and inhibitor specificity of the Lac carrier. It was hoped that in the process, we would develop useful reagents and derive valuable insights into the nature of this vital transport system that would serve us well in future contemplated studies.

Materials and Methods

Incubation Media

The standard medium used for preparing cells had the following composition (in mM): 137 NaCl, 5 KCl, 2 Na L-lactate, 1 $CaCl_2$, 0.5 $MgCl_2$, 5.6 glucose, 5 HEPES buffer, pH 7.40, and 1 mg/ml of crystalline bovine serum albumin. The experimental media for the [^{14}C]lactate influx assays, wherein the affinity of various compounds for the Lac carrier was determined, were slightly modified as follows. Compounds were evaluated as potential substrate analogues at constant extracellular Cl^- (125 mM) over a concentration range of 1–20 mM (equimolar substitution of glucuronate, an inert replacement anion [32]). Studies were conducted in the presence of 1 mM lactate to reduce competition. Thus, the medium in which transport assays were conducted contained 117 mM NaCl, 1 mM Na L-lactate, and 0–20 mM Na glucuronate varying reciprocally with Na salts of the test anions in addition to the normal concentrations of KCl, $CaCl_2$, $MgCl_2$, glucose, HEPES, and albumin as stated above. In order to preserve isotonicity, all of the media were adjusted to a total osmolarity of 305 ± 2 mOsm/liter, as monitored by a vapor pressure osmometer (model 5500, Wescor).

Neutrophils

Human peripheral neutrophils were isolated by sequential dextran sedimentation at 37°C followed by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation at room temperature [4]. Contaminating erythrocytes were removed by hypotonic lysis in distilled water for 20 sec. The neutrophils were washed three times and then counted. The purity of the neutrophil suspensions averaged 98% as assessed by eosin Y exclusion, and was not affected by any of the agents or incubation conditions tested. The cells were kept in the standard medium (2 mM lactate, 145 mM Cl^-) for 1 h at 37°C prior to experimentation. Studies were initiated by pelleting the cells and immediately resuspending them in the transport medium containing [^{14}C]lactate together with the compound to be tested. All assays were carried out at 37°C.

Reagents and Chemicals

All inorganic salts were obtained from Fisher Scientific, St. Louis, MO. The following reagents were purchased from Sigma Chemical, St. Louis, MO: crystalline bovine serum albumin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(N-morpholino)ethanesulfonic acid (MES), N-ethylmaleimide (NEM), L-lactic acid, sodium lactate, sodium glucuronate, D-glucuronic acid, D-glucose, and mersalyl. The various inhibitors as well as aliphatic and aromatic carboxylic acids, too numerous to cite individually, were each bought from either Sigma; Aldrich Chemicals, Milwaukee, WI; Pierce Chemical, Rockford, IL, or Fluka. L-[^{14}C]lactic acid was purchased from New England Nuclear, Boston, MA: the specific activity was 90 mCi/mmol.

One-Way [^{14}C]Lactate Fluxes

The incubations were performed at 37°C in capped, plastic tubes (Falcon Plastics, Oxnard, CA) under various experimental conditions (neutrophils 8–12 × 10^6/ml). Influx experiments were performed in the presence of [^{14}C]lactate (3.0 μCi/ml). At 1- and 2-min intervals, triplicate aliquots of the cell suspensions were layered on 0.5 ml of silicone oil (Versilube F-50, General Electric, Waterford, NY) contained in 1.5 ml plastic tubes and centrifuged for 1 min at 8,000 × *g* in a microcentrifuge (Beckman Instruments, Fullerton, CA). Cell separation occurred in <5 s. The aqueous and oil phases were aspirated and discarded. The neutrophil pellets were excised and counted in a liquid scintillation counter (Tri-Carb 1500, Packard Instruments). Influx rates were computed by least-squares fitting the cell label data *vs.* time to a single exponential equation as previously described [32]. The final steady-state uptake was taken as 5.2 mM. This is the value determined experimentally in previous studies using a chemical measurement (LDH assay) for the intracellular lactate content [32]. The initial rate of [^{14}C]lactate influx from a 1 mM lactate medium (pH 7.40) into control cells was 0.64 ± 0.09 meq/liter · min.

pH-Stat Methods

H^+ movements across the cells were monitored for changes in the pH of the bathing solutions using conventional pH-stat techniques. For these experiments, an extracellular pH (pH_o) of 6.0 was selected in order to accelerate fluxes via the carrier [32] and to provide a favorable inward driving force for H^+ and the various anionic substrates. Another advantage is that any potential uptake of the various substrate analogues through the Cl^-/HCO_3^- exchanger was eliminated since this degree of extracellular acidification abolishes anion exchange [27]. The buffering power of the medium was reduced by lowering the concentration of MES from 5 to 0.1 mM. The neutrophils (6–8 × 10^6/ml) were exposed to the different compounds in a 6 ml reaction chamber that was maintained at 37°C. The pH_o was kept at 6.0 by the frequent injection of μl quantities of acid by means of a microprocessor-controlled automatic buret (Metrohm pH-stat system 1, Sybron/Brinkmann Instruments, Des Plaines, IL) whose output was connected to a chart recorder. The results, determined from the total amount of HCl added and the number of neutrophils in the reaction mixture, were expressed as nanomoles of $H^+/10^6$ cells. These units were then converted to meq/liter of cell water for the corresponding H^+ influx into the neutrophils by multiplying by a factor of 3.65 based on a cell water volume of 0.274 picoliters/cell [30]. The baseline uptake of H^+ equivalents from the lactate-free and 2 mM L-lactate media at pH_o 6.0 amounted to 1.6 ± 0.2 and 12.1 ± 0.9 meq/liter · min, respectively.

Controls for each day included medium alone, 0.25 mM mersalyl (a saturating dose to abolish transport through the Lac carrier [32]), 2 mM lactate, and 2 mM lactate + 0.25 mM mersalyl. Test compounds were examined in the absence of lactate, with and without mersalyl in an effort to assess the differing contributions of carrier-mediated transport (mersalyl-sensitive component) *vs.* simple nonionic diffusion (mersalyl-resistant pathway).

Table 1. Effect of sulfhydryl-reactive agents on [^{14}C]lactate influx into human neutrophils

Compound	Apparent $K_{0.5}$ (mM)
Mersalyl	0.011 ± 0.003
p-Chloromercuribenzoate (PCMB)	0.021 ± 0.004
p-Chloromercuribenzenesulfonate (PCMBS)	0.024 ± 0.005
N-Ethylmaleimide (NEM)	0.039 ± 0.007
5,5'-Dithiobis(2-nitrobenzoate) [DTNB]	0.27 ± 0.07
2,2'-Dithiobispyridine (Aldrithiol-4)	0.21 ± 0.05
4,4'-Dithiobispyridine	0.35 ± 0.08
Eosin-5-isothiocyanate	0.87 ± 0.25
Eosin-5-maleimide	2.0 ± 0.3
Maleic anhydride	1.9 ± 0.9
Iodoacetate	4.0 ± 1.7

Table 2. Ability of sulfhydryl-reactive agents to cause irreversible inhibition of the Lac transporter

Drug	Concentration (mM)	% Inhibition of Lactate Influx
NBD-Cl[a]	1.0	96 ± 6
	0.5	86 ± 9
	0.25	81 ± 11
	0.125	70 ± 10
Mersalyl	0.25	82 ± 8
2,2'-Dithio-bispyridine	0.5	66 ± 12

Neutrophils were exposed to the various drugs at the stated concentrations for 30 min at 4°C. Thereafter, the cells were washed and resuspended in drug-free medium for measurement of [^{14}C]lactate influx at 37°C. Influx rates into cells pretreated with drugs were determined relative to that into normal neutrophils and results expressed as the % inhibition of control influx. Data are from four experiments.

[a] NBD-Cl = 7-chloro-4-nitrobenz-2-oxa-1,3-diazole.

Data Analysis

The apparent $K_{0.5}$ values for suppression of [^{14}C]lactate influx from a 1 mM lactate medium were determined by least-squares fits of a Michaelis-Menten inhibition equation to the dose-response data for a given compound as previously described [32]. Results represent the means ± SEM of three to five separate experiments with cells from different donors.

For the pH-stat experiments, compounds were added at an arbitrary concentration equal to one-sixth of their apparent $K_{0.5}$ values for inhibition of [^{14}C]lactate influx. This concentration, rather than a fixed dose for all, was selected so as to maintain an equivalent degree of saturation of external binding sites under each treatment condition. This choice facilitated direct comparisons between the data sets for the different substrates. The H^+ influxes were found to be linear over the course of the first 1 min; the rates were determined from the slopes of the linear regressions. The transport rate of a given compound via the Lac carrier relative to that for L-lactate was obtained from the ratio of the mersalyl-sensitive flux in the presence of the substrate analogue over that with 2 mM L-lactate.

The ability of any of the organic anions to permeate as the free acid via nonionic diffusion was quantitatively expressed by measuring the associated H^+ influx in the presence of the compound + mersalyl over and above that with mersalyl alone where no diffusional entry of H^+ equivalents takes place.

Results

Inhibitors

The apparent $K_{0.5}$ values for inhibition of [^{14}C]lactate influx by a variety of sulfhydryl-reactive compounds are given in Table 1. By far, the most potent chemical class of agents was the organomercurials. The three members of this series were roughly equipotent, mersalyl, PCMB, and PCMBS each blocking lactate influx with a $K_{0.5}$ of ~10–25 μM, a value one to two orders of magnitude lower than for most of the other drugs tested. NEM, DTNB, and the two bifunctional bispyridines also demonstrated considerable activity whereas eosin-5-isothiocyanate, eosin-5-maleimide, maleic anhydride, and iodoacetate all exhibited rather modest suppressive effects. It is important to point out that in our standard assays, cells were exposed to the compounds at zero time along with the radiolabeled tracer and, therefore, that no preincubation period with the drugs was routinely employed.

Due to the fact that these agents bear reactive groups capable of covalent interaction with SH groups on the Lac carrier protein, we examined a number of these compounds for their ability to irreversibly block transport function. Since an effective inhibitor will necessarily lead to the intracellular accumulation of lactic acid with consequent acidification, we sought to divorce direct effects of the drug on the Lac transporter from indirect effects related to the buildup of lactic acid. This aim was accomplished by pretreating cells with drugs for 30 min at 4°C, a maneuver which suppresses glycolysis and, therefore, the production and secretion of lactic acid. After the pretreatment period, the cells were washed twice, resuspended in drug-free medium, and then assayed for the uptake of [^{14}C]lactate at 37°C. The results of a series of experiments are presented in Table 2. Under these specified conditions, NBD-Cl (whose activity at 37°C could not be adequately evaluated because of toxicity at higher con-

centrations) proved to be the most active, completely abolishing lactate influx in an irreversible manner at 1 mM. Mersalyl and 2,2'-dithiobispyridine, while efficacious, were only partially so, achieving incomplete inhibition to the extent of 82 and 66% at concentrations of 0.25 and 0.5 mM, respectively. Raising the concentration to 1 mM had no further enhancing effect nor did extending the drug pretreatment period with cells to 60 min. In contrast, with regard to the ability to cause irreversible inhibition, DTNB at 0.5 mM was essentially inert.

Substrate Selectivity
Unsubstituted Organic Anions

The selectivity of the Lac carrier is sufficiently low that broad acceptance of even unsubstituted, straight chain aliphatic monocarboxylates occurs (Table 3). In the series shown, there is a definite trend towards higher affinities with increasing carbon-chain length. Thus, the apparent $K_{0.5}$ fell from 29.1 mM with formate to 4.5 mM with valerate. *N*-propionate, *n*-butyrate, and formate were transported by the Lac carrier though the rates were only a small fraction (0.41, 0.32, and 0.17, respectively) of that for L-lactate; acetate and *n*-valerate were not transported at all. *N*-valerate, presumably a competing subtrate, is one of several potentially interesting compounds (*see* discussion of Table 4 below) that binds with ~3-fold higher affinity than L-lactate yet is not transported.

It should also be pointed out that each of these unsubstituted monocarboxylates could be shown to permeate across the plasma membrane via nonionic diffusion. Normalizing all the concentrations to that of formate (assuming simple diffusion to be directly proportional to concentration) gave a relative rank order of diffusional permeability of 1.0 : 1.8 : 3.2 : 7.7 : 14.2 in going from formate to valerate. These results are in keeping with enhanced lipid solubility with increasing carbon atom content.

Physiologic Anions

We also evaluated the ability of a number of physiologically relevant organic anions to bind to and be transported by the Lac carrier (Table 3). With respect to half-saturation constants for inhibition of lactate influx, none exhibited a higher affinity than did L-lactate ($K_{0.5}$ = 11.3 mM). Pyruvate, glycolate, D-3-hydroxybutyrate, glyoxalate, and acetoacetate all displayed apparent $K_{0.5}$ values (12.7, 13.6, 9.4, 15.4, and 14.2 mM, respectively) similar to that for L-lactate, while glycerate and 2-oxobutyrate were less active ($K_{0.5}$ = 22.0 and 24.0 mM). None permeated via nonionic diffusion to any significant degree, although each was capable of being transported inward via the Lac cotransport system at rates which for some approached that for L-lactate. Succinate, malonate, and 2-oxoglutarate, three dicarboxylates, and citrate, a tricarboxylate, were essentially devoid of affinity, none having any effect on lactate influx at a concentration of 20 mM. The inorganic phosphate ion likewise had no measureable activity at 20 mM.

Cinnamate Derivatives

Several cinnamate (phenylpropenoic = phenylacrylate) and related indole compounds were tested (Table 4) including CHC, the classical inhibitor of monocarboxylate transport in mitochondria as first described by Halestrap and Denton [15, 17, 18]. Of these, UK-5099, as previously reported [32], proved to be the most potent with an apparent $K_{0.5}$ of 41 μM, three orders of magnitude lower than that of the parent compound CHC. The latter is a much weaker inhibitor of lactic acid transport in neutrophils than a blocker of monocarboxylate transport in mitochondria [18], the affinities differing by ~3 orders of magnitude. It should also be stressed that each of the analogues listed has been found to suppress Cl^-/HCO_3^- exchange in neutrophils with $K_{0.5}$ values similar to those for inhibition of lactate fluxes [27, 32]. UK-5099 as well as the 2-phenyl-, 2-fluoro-, and 2-cyanocinnamate derivatives are not transported via the Lac carrier to any appreciable extent, although all three readily diffuse across the plasma membrane as free acids. The properties of nontransportability by the Lac cotransport system but permeation via nonionic diffusion seem to be general characteristics of all compounds bearing the large, hydrophobic phenyl and indole groups (*see also,* 3-indoleacrylate).

Acetate Derivatives

Nine substituted acetates, namely chloro, hydroxy (glycolate), oxo (glyoxalate), aminooxo (oxamate), bromo, phenyl, phenylglycolate (mandelate), aceto, and thio (thioglycolate), each displayed apparent $K_{0.5}$ values comparable to or lower than L-lactate (Tables 3 and 4). Phenylglyoxalate, a di-substituted derivative, was the sole exception, displaying considerably less affinity. While evidently possessing

Table 3. Unsubstituted aliphatic 1-monocarboxylates and physiologic organic anions as potential substrates

Anion	Apparent $K_{0.5}$ (mM)	Pathways of entry	
		Nonionic diffusion[a]	Transport via Lac carrier (rate relative to L-lactate)
Unsubstituted 1-Monocarboxylates			
Formate	29.1 ± 10.4	2.39 ± 0.31	0.17
Acetate	22.1 ± 5.1	3.27 ± 0.96	0.06
n-Propionate	17.9 ± 3.5	4.77 ± 0.65	0.41
n-Butyrate	8.2 ± 1.0	5.18 ± 0.79	0.32
n-Valerate	4.5 ± 0.6	5.25 ± 0.84	0.00
Physiologic Anions			
Glycolate (hydroxyacetate)	13.6 ± 3.3	None	0.55
L-Lactate	11.3 ± 1.5	None	1.00
Pyruvate	12.7 ± 3.2	None	0.93
Glyoxalate (oxoacetate)	15.4 ± 2.9	None	0.81
Glycerate (2,3-dihydroxypropionate)	22.0 ± 5.4	None	0.69
2-Oxobutyrate	24.0 ± 5.7	None	0.85
Acetoacetate (3-oxobutyrate)	14.2 ± 3.3		0.35
2-Oxoglutarate (2-oxo-1,5-pentanedicarboxylate)	—[b]		
Malonate (methanedicarboxylate)	—[b]		
Succinate (1,4-butanedicarboxylate)	—[b]		
Citrate (2-hydroxy-1,2,3-propanetricarboxylate)	—[b]		

[a] Nonionic diffusion stands for the mersalyl-resistant component of the H^+ influx rate (in meq/liter · min) in the presence of a given compound. None indicates that no significant influx could be detected with a compound + mersalyl over that with mersalyl alone.
[b] No inhibition at 20 mM.

groups favorable for binding to the Lac carrier with relatively high affinity, the short two carbon backbone may be somewhat inferior to the three carbon propionate moiety in conferring transportability as some of these compounds (e.g., 2-chloroacetate and oxamate; *see* also Table 5) were transported inward at a rate only a small fraction of that for L-lactate or their propionate counterparts. These compounds, along with benzoylformate (*see* Table 4, Other Compounds), may be potentially useful in future studies as non or poorly transportable substrate analogues that presumably compete with L-lactate for the same translocation site on the carrier. They suppress lactate influx with activities comparable to or greater than L-lactate itself, yet do not permeate via nonionic diffusion.

Propionate Derivatives

Of the additional compounds listed in Table 4 (pyruvate and glycerate have already been mentioned in Table 3), 3-indolepropionate and 3-chlorolactate displayed K_i values (13.0 and 13.7 mM) similar to L-lactate, whereas 3-phenyllactate (41.5 mM) was considerably less potent. Surprisingly, thiolactate, the thio analogue of lactate, blocked [^{14}C]lactate influx with a $K_{0.5}$ of only 3.9 mM, thereby implying a ~3-fold greater affinity than L-lactate. On the other hand, the SH group evidently prevented transport by the Lac carrier as only a very small H^+ influx could be detected by pH-stat techniques. For thiolactate, mersalyl could not be used to dissociate the carrier-mediated H^+ flux from nonionic diffusion due to interactions between the drug and the thiol

Table 4. Survey of miscellaneous anions

Anion	Apparent $K_{0.5}$ (mM)	Pathways of entry	
		Nonionic diffusion[a]	Transport via Lac carrier (rate relative to L-lactate)
Cinnamate Derivatives			
UK-5099[b]	0.041 ± 0.010	2.1 ± 0.2	0.00
2-Phenylcinnamate	0.80 ± 0.21	10.2 ± 1.4	0.08
2-Cyanocinnamate	7.7 ± 2.0	11.1 ± 2.5	0.11
2-Cyano-4'-hydroxycinnamate (CHC)	3.2 ± 0.9		
3'-Bromocinnamate	4.2 ± 0.5		
3-Indoleacrylate	4.7 ± 0.3	20.8 ± 5.1	0.03
2-Fluorocinnamate	6.4 ± 1.0	11.9 ± 2.7	0.04
4'-Chlorocinnamate	6.9 ± 2.0		
2-Cyano-3'-hydroxycinnamate	9.8 ± 2.6		
2-Methylcinnamate	12.9 ± 4.1		
trans-Cinnamate	15.3 ± 5.9		
Acetate Derivatives			
Bromoacetate	4.7 ± 1.2		
Chloroacetate	6.4 ± 1.3	None	0.43
Phenylacetate	10.2 ± 1.7	14.3 ± 4.0	0.19
Oxamate (aminooxoacetate)	4.9 ± 0.6	None	0.25
Thioglycolate	7.3 ± 1.3		
Mandelate (phenylglycolate)	10.1 ± 2.5		
Phenylglyoxalate	40.5 ± 19.3		
Propionate Derivatives			
3-Indolepropionate	13.0 ± 1.6		
3-Chlorolactate	13.7 ± 2.1	None	0.65
Thiolactate	3.9 ± 0.5		<0.15
3-Phenyllactate	41.5 ± 17.8		
Other Compounds			
Niflumate	0.62 ± 0.11	7.5 ± 1.6	0.12
Flufenamate	0.72 ± 0.21		
n-Butylmalonate	1.5 ± 1.0		
1,2,3-Benzenetricarboxylate	2.6 ± 1.9		
Cyclohexanecarboxylate	9.3 ± 2.4	13.4 ± 3.5	0.12
Benzoylformate	13.6 ± 2.8	None	0.04
2-Hydroxy-*n*-valerate	24.3 ± 8.0		
2-Hydroxyisobutyrate	33.3 ± 13.0		
p-Aminohippurate	42.3 ± 21.3		

[a] Nonionic diffusion stands for the mersalyl-resistant component of H^+ influx (in meq/liter · min) for a given compound.
[b] UK-5099 = 2-cyano-3-(1'phenylindol-3'-yl)acrylate was a generous gift of Pfizer Central Research Laboratories of Sandwich, Kent, UK.

group of the compound. However, the total H^+ influx rate with thiolactate over that with medium alone (1.6 ± 0.3 meq/liter · min) as compared to L-lactate (10.5 ± 1.1 meq/liter · min) implies that even if all of the flux could be ascribed to transport via the Lac carrier then the transport rate would be at most 0.15 that of L-lactate.

Miscellaneous Other Compounds

Of the remaining organic anions (Table 4), niflumate and flufenamate blocked [^{14}C]lactate influx with apparent $K_{0.5}$ values of 0.62 and 0.72 mM, respectively. *n*-Butylmalonate and 1,2,3-benzenetricarboxylate, inhibitors of the mitochondrial di- and tricarboxylate

Table 5. Effect of various substituents at positions 2, 3, and 4 of the acetate, propionate, and butyrate backbones

Group	Apparent $K_{0.5}$ (mM)	Transport via Lac carrier (rate relative to L-lactate)
Propionate Derivatives		
D,L-2-Hydroxy (Lactate)	35.1 ± 9.3	0.71
3-Hydroxy	39.3 ± 17.9	0.50
2-Oxo (Pyruvate)	12.7 ± 3.2	0.93
2-Amino	42.4 ± 16.8	0.00
2-Methyl (Isobutyrate)	13.5 ± 4.1	0.31
2-Phenyl	14.3 ± 2.1	0.10
3-Phenyl	8.5 ± 1.3	
2-Nitro	—[a]	
2-Chloro	6.9 ± 1.1	0.61
3-Chloro	10.2 ± 2.2	0.34
Acetate Derivatives		
2-Hydroxy (Glycolate)	13.6 ± 3.3	0.55
2-Oxo (Glyoxalate)	15.4 ± 2.9	0.81
2-Methyl (Propionate)	17.9 ± 3.5	0.41
2-Phenyl	10.2 ± 1.7	0.19
2-Chloro	6.4 ± 1.3	0.43
Butyrate Derivatives		
2-Hydroxy	30.8 ± 5.4	0.88
3-Hydroxy	16.2 ± 4.4	0.64
4-Hydroxy	28.0 ± 10.8	0.42
2-Phenyl	7.9 ± 1.4	0.00
3-Phenyl	16.1 ± 4.1	
4-Phenyl	18.2 ± 3.5	

[a] No inhibition at 20 mM.

carriers [20], displayed $K_{0.5}$ values of 1.5 and 2.6 mM. Whether or not these compounds actually bind to the substrate site of the Lac transporter and therefore represent true competitive inhibitors of lactate was not addressed.

Nature and Position of Substituents

Table 5 shows data on the effect of various substituents at the 2-position of the *n*-propionate and acetate backbones. As all of the propionate compounds were supplied as racemic mixtures of the D- and L-forms, comparisons have been made to D,L-lactate rather than to the pure L-isomer. With respect to binding to the external translocation site of the Lac carrier, the selectivity sequence in terms of decreasing affinities was as follows: Cl > oxo ~ CH_3 ~ phenyl > OH ~ NH_2 ≫ NO_2. This pattern is similar to the rank order of these substituents at the 2-position of acetate: Cl > phenyl > OH ~ oxo ~ CH_3. With regard to transportability, however, a somewhat different picture emerges. Starting with the most favorable group, the sequence for the propionate compounds was: oxo > OH ~ Cl > CH_3 ≫ phenyl ~ amino as compared to oxo > OH > Cl ~ CH_3 > phenyl for the acetate series.

Table 5 also indicates the effect of relative position on the molecule of a given group. For this analysis, we examined the alterations that ensued when chloro, hydroxy, or phenyl groups were systematically moved from the 2 to the 3 or 4 positions of propionate and butyrate. From inspection of the data of Table 5, several trends are evident. For both propionate and butyrate compounds, the half-saturation constants for inhibition of L-lactate influx were little affected by varying the position of a given substituent, the apparent $K_{0.5}$ values for any set always falling within a factor of two of each other. There were no consistent differences in relative affinities for the 2- as compared to the 3- or 4-positions. Regarding transportability, however, there was indeed a marked preference for the 2-position. This feature is clearly seen for the 2- *vs.* 3-hydroxy- and chloro-substituted propionate derivatives as well as for the 2- *vs.* 3- *vs.* 4-hydroxybutyrate derivatives. Unfortunately, similar comparisons could not be made with phenyl substituents as none of the compounds bear-

Table 6. Aromatic Monocarboxylates

Additions to benzoate backbone or other compounds	Apparent $K_{0.5}$ (mM)	Entry via Nonionic diffusion[a]
None	14.9 ± 4.2	
2-Chloro	13.9 ± 2.9	
3-Chloro	5.3 ± 0.7	26.2 ± 3.7
4-Chloro	4.8 ± 0.5	
2-Hydroxy (Salicylate)	12.6 ± 3.7	29.3 ± 3.0
3-Hydroxy	20.9 ± 7.2	
4-Hydroxy	16.5 ± 4.5	
2-Methyl	10.1 ± 2.4	
3-Methyl	8.3 ± 2.1	33.4 ± 6.5
4-Methyl	17.3 ± 3.5	
2-Nitro	—[b]	
3-Nitro	29.2 ± 10.6	
4-Nitro	18.5 ± 3.3	
2-Amino	22.7 ± 5.5	
3-Amino	22.8 ± 4.6	
4-Amino	24.2 ± 6.5	
Benzenesulfonate	9.1 ± 1.7	
1-Naphthoate (1-Naphthalenecarboxylate)	5.3 ± 1.3	12.7 ± 1.8
2-Naphtholate (2-Naphthalenecarboxylate)	1.2 ± 0.2	
Pyrazinoate (2-Pyrazinecarboxylate)	10.9 ± 2.4	
Picolinate (2-Pyridinecarboxylate)	8.6 ± 0.9	
Nicotinate (3-Pyridinecarboxylate)	21.7 ± 9.0	
Isonicotinate (4-Pyridinecarboxylate)	15.9 ± 2.8	

[a] Nonionic diffusion stands for the mersalyl-resistant component of H^+ influx (in meq/liter · min) for a given compound.
[b] 19% inhibition at 20 mM.

ing this particular group was transported via the carrier.

Aromatic Anions

Table 6 lists the apparent K_i values for a series of aromatic monocarboxylates, all substituted benzoate derivatives. At the outset, it is important to bear in mind that the parent compound, namely the unsubstituted benzoate molecule, possesses a $K_{0.5}$ of 14.9 mM, similar to that of L-lactate itself. The relative rank order in terms of decreasing affinities of the various mono-substituents (Cl, OH, CH_3, NH_2, and NO_2) at the 2-, 3-, and 4-positions of the benzene ring was as follows (H standing for no substituent):
at the 2-position,
$CH_3^- \; OH^- \; Cl^- \; H > NH_2 \gg NO_2$
at the 3-position,
$Cl^- \; CH_3 > H^- \; OH^- \; NH_2 > NO_2$
at the 4-position,
$Cl > H^- \; OH^- \; CH_3^- \; NO_2^- \; NH_2$.
In general, then,
$Cl > H^- \; OH^- \; CH_3 > NH_2 > NO_2$.
Mono-Cl-substituted derivatives inhibit the Lac transporter with slightly higher affinity than H while OH and CH_3 derivatives are roughly equipotent to benzoate itself. The more polar nitro and amino moieties are distinctly detrimental to activity, reducing potency by a factor of two or more.

As a rule, the relative position of a given substituent on the benzene ring was not a major factor in determining potency, all generally falling within a factor of two of each other. When tested using the pH-stat device, neither the 3-Cl, 2-OH, nor 4-CH_3 analogues was transported inward via the Lac carrier to any significant extent as evidenced by the lack of mersalyl-sensitive proton flux. On the other

hand, each compound was nonetheless capable of crossing the plasma membrane in the free acid form (i.e., in association with H^+) since an alkalinization of the bathing medium could be easily detected. As we could measure no mersalyl-sensitive component to the H^+ influx, it would appear that the mechanism of entry into the cytosol is, in all likelihood, nonionic diffusion of these highly lipophilic compounds.

Several other aromatic monocarboxylates and sulfonate derivatives were also examined (Table 6). The affinity of benzenesulfonate was comparable to that for benzoate ($K_{0.5}$ values of 9.1 and 14.9 mM, respectively), implying that the nature of the charged moiety, whether carboxyl or sulfonate group, is relatively unimportant. This inference is also supported by the previously alluded to finding that PCMB and PCMBS block [^{14}C]lactate influx with closely similar $K_{0.5}$ values (Table 1). The 1- and 2-naphthalenecarboxylate compounds suppressed lactate transport with apparent $K_{0.5}$ values of 5.3 and 1.2 mM that are clearly lower than for L-lactate. These compounds were not transported to any measureable degree, but did gain access to the cytoplasm via nonionic diffusion. Pyrazinoate, picolinate, nicotinate, and isonicotinate each blocked lactate uptake with affinities roughly equal to those for benzoate and lactate itself.

Discussion

In contrast to the situation in red blood cells [10], lactate does not permeate via the Cl^-/HCO_3^- exchange mechanism in human neutrophils [32]. This is due to a lack of affinity which presumably also holds true for closely related analogues. Moreover, permeation of lactic acid and other physiologically relevant substrates via nonionic diffusion of the undissociated free acid form does not occur to any appreciable extent in neutrophils [32 and Table 3]. Thus, the Lac carrier constitutes the sole pathway for the movement of lactate ions across the plasma membrane of these cells.

Inhibitor Profile

In conformity with most other monocarboxylate carriers described to date in mammalian cells [1, 6, 8, 11, 12, 18, 21, 34, 35], the neutrophil Lac transporter [32] is strikingly sensitive to organomercurials and to cyanocinnamate derivatives. The Na^+-dependent Lac cotransport system of the kidney is an exception, being resistant to the mercurials [37] while being blocked by CHC [26]. The uniform sensitivity of H^+ + lactate$^-$ cotransporters to organomercurials, notably mersalyl, PCMB, and PCMBS, has been taken to imply the involvement of a critical sulfhydryl group in the transport reaction. Indeed, a variety of thiol compounds and other SH-reactive agents have also been found to block transport in one or more cell types [8, 12, 34, 35]. Of the drugs tested in our system, NEM, DTNB, 2-2′-dithiobispyridine, and NBD-Cl, which fall into this category are probably the most useful by virtue of enhanced potency and/or irreversible inhibition. The relatively high potency of NEM and NBD-Cl as compared to iodoacetate echoes the pattern seen for monocarboxylate transport in erythrocytes [10].

It is noteworthy that PCMB and PCMBS block lactate fluxes with an apparent $K_{0.5}$ of ~20 μM, while their congeners lacking the organic mercury group (i.e., 4-chlorobenzoate and benzenesulfonate) display inhibition constants of ~4–9 mM. These values are remarkably two orders of magnitude greater, signifying a vast reduction in apparent affinity. Conceivably, the physico-chemical properties of the bulky, hydrophobic Hg atom are directly responsible for creating the requisite complementary interactions at the binding site although indirect effects on the pK′ of the carboxylic acid group may also play a role.

In the cyanocinnamate series, plasma membrane carriers such as those of neutrophils, erythrocytes, and other cells are much less sensitive to the parent compound CHC [8, 16, 29, 32], the classical inhibitor originally reported by Halestrap and Denton [17], than is the comparable system in mitochondria. In this regard, the neutrophil transporter seems to be particularly resistant, the $K_{0.5}$ of 3.2 mM being one to three orders of magnitude greater than for human erythrocytes (0.06 mM [16]), guinea pig cardiac myocytes (0.04 – 0.10 mM [23, 24]), rat hepatocytes (0.11 mM [12]), Ehrlich ascites tumor cells (0.5 mM [34]), and rat mitochondria (0.006 mM [18]). The relatively low apparent affinity of the neutrophil Lac carrier may relate in part to the presence of albumin (in our studies) which is known to bind CHC [18], although a higher degree of intrinsic resistance to this series of compounds seems likely. However, several analogues and related derivatives display considerable increases in potency, 2-phenylcinnamate and UK-5099 being the two of greatest practical application. These drugs behave as reversible although nonspecific inhibitors of lactate transport since they also block Cl^- fluxes through the Cl^-/HCO_3^- exchanger in both red blood cells and neutrophils [1, 8, 16, 27, 29]. While incapable of being transported by the neutrophil Lac carrier, these compounds do gain access to the cell interior by way of nonionic diffusion [33], an attribute which detracts from their general usefulness. On the other

hand, the weak blocker CHC has been reported not to enter neutrophils [29] or Ehrlich ascites tumor cells [34], but is taken up by erythrocytes [18]. The fact that albumin, which binds CHC, prevents uptake into erythrocytes [18] may explain some of these apparent disparities since our studies were always performed in the presence of 0.1% albumin.

Substrate Specificity

From an analysis of the competitive interactions between [^{14}C]lactate and a variety of potential analogues as reported herein, a picture emerges of the substrate binding site of the Lac carrier of human neutrophils. The salient features of the external translocation site may be briefly summarized as follows:

(a) With respect to aliphatic monocarboxylates (either straight chain or branched), a number of unsubstituted as well as substituted analogues are bound and subsequently transported.

(b) For unsubstituted alkyl monocarboxylates, affinities progressively rise with increasing carbon atom content, at least up to five. While unsubstituted organic anions are accepted, the carrier displays a preference for substituted compounds, mono- and di-substituted analogues exhibiting more activity provided they bear favorable replacement groups.

(c) Cl, OH, oxo, CH_3, and phenyl groups are associated with higher affinity than H while the more polar NH_2 and NO_2 groups are detrimental to activity. Substituents at position 2 are readily transported as are those at position 3, if only somewhat slower. Compounds containing phenyl groups are not transported by the carrier although they cross the plasma membrane via nonionic diffusion.

(d) Replacement of an O atom by S (e.g., thiolactate or thioglycolate) causes no change in affinity, but prevents translocation.

(e) Aromatic monocarboxylates are bound, but not transported. Among benzoate derivatives, Cl and OH substituents confer higher affinity than do NH_2 and NO_2. Although not as extensively studied, aromatic sulfonate derivatives bind with affinities comparable to their carboxylate counterparts. The relative position of a given substituent on the benzene ring is of minor importance.

(f) Di- and tricarboxylates do not bind.

The constellation of features that describes the Lac carrier of neutrophils compares quite favorably with the substrate selectivity of the monocarboxylate transporter of erythrocytes that has been extensively studied by Deuticke and colleagues [5–10]. Many similarities exist relating to the broad acceptance of a large variety of organic anions as noted above. These findings imply functional and structural conservation of the key elements of the substrate binding site of these presumably homologous membrane transport proteins. Nonetheless, differences appear to exist though these seem to be more in the nature of minor details rather than fundamental distinctions. For example, (i) thiolactate and thioglycolate are bound by the neutrophil system in a manner comparable to their oxygen-containing counterparts whereas substitution of an S atom for O results in a marked reduction in affinity for the erythrocyte carrier [8]. (ii) In neutrophils, for propionate and butyrate analogues, substituents at positions 2 and 3 display roughly similar affinities and transportability whereas in red blood cells, 3-substituted compounds are poorly transported, if at all [8]. (iii) The most striking difference between the erythrocyte and neutrophil systems is the lack of affinity of the former for formate, glyoxalate, oxamate, glycerate, isobutyrate, 2-hydroxyisobutyrate, and N-formylglycine [8, 23]. The data of Tables 3–5 demonstrate convincingly that these compounds are readily bound and transported across the neutrophil plasma membrane.

The nature of the substituent is important in determining specificity. In both erythrocytes and neutrophils, strongly electron-withdrawing groups such as oxo and Cl are associated with relatively higher degrees of affinity than OH. As pointed out by Deuticke [8], the acidity of the carboxyl group is probably involved in these patterns since acids with lower pK′ values are usually better substrates. A similar observation was also made by Ullrich et al. [38] who analyzed the issue of specificity of the Na^+ + lactate$^-$ cotransport system in the renal proximal tubule. They found that specificity was related to many factors including molecular size, hydrophobicity, electron-attracting abilities of substituents, and charge distribution on the molecule. Ullrich et al. [38, 39] emphasized the primary importance of the charge situation in the neighborhood of the carboxylic moiety, electron-attracting groups tending to increase the acidity of the compound and the stability of the anion. This effect decreases with distance from the reaction center; i.e., the free carboxylic group.

Pronounced differences exist among various carriers with respect to relative affinities for lactate and pyruvate and to stereoselectivity. Most transporters (those of erythrocytes, hepatocytes, and cardiac myocytes [8, 12, 23, 24]) display a considerably higher affinity (three- to tenfold) for pyruvate over lactate whereas neutrophils, Ehrlich ascites tumor cells [34], and sarcolemmal vesicles [36] have been reported to exhibit approximately equal affinities for the two natural substrates. It is of note, however,

that the absolute value for K_m (lactate) in neutrophils (11–13 mM [32]) is four- to sevenfold higher than in red cells, hepatocytes, and cardiac myocytes [8, 12, 23, 24]. Also, while erythrocytes and hepatocytes demonstrate almost complete stereoselectivity for L- over D-lactate [8, 12, 16, 23], neutrophils and guinea pig heart cells [24] show only two- to threefold greater preference for the L-form, whereas in Ehrlich tumor cells [34] the two forms have identical binding constants.

For certain analogues of pyruvate, little uniformity seems to exist among the well-studied systems. For example, glyoxalate and oxamate are poor substrates in erythrocytes [8, 23]. In myocytes [23], however, glyoxalate constitutes a poor substrate, but oxamate binds with similar affinity to lactate, whereas in neutrophils glyoxalate and lactate are similar, but oxamate binds with two- to threefold higher affinity. On the other hand, 2-oxobutyrate possesses 11- and 17-fold higher affinities than lactate in erythrocytes [23] and cardiac myocytes [23], but twofold lower in neutrophils, whereas 3-oxobutyrate binds with roughly similar affinity to lactate in erythrocytes and neutrophils, but with sixfold higher affinity in myocytes.

In general, the Na^+-dependent, SH-reagent-insensitive system of the kidney proximal tubule brush border [19, 22, 37–40] has a specificity that largely resembles that of the Na^+-independent, SH-reagent-sensitive system of red cells [1, 6–8]. There are, however, a few small differences: the red cell has a high affinity for 3-hydroxypropionate and 4-hydroxybutyrate and a very low affinity for 2-hydroxyisobutyrate, whereas the kidney behaves in the opposite fashion. From the data presented herein, it would appear that the neutrophil Lac carrier more closely mimics that of erythrocytes, another Na^+-independent Ha^+ + lactate$^-$ cotransport system. The monocarboxylate carriers of red cells, kidney proximal tubules, and neutrophils are all capable of transporting simple unsubstituted fatty acids albeit slowly [8, 38], while that of Ehrlich ascites tumor cells apparently does not [34].

With respect to aromatic monocarboxylates, 1- and 2-naphthoate are considerably more inhibitory in neutrophils than nicotinate and isonicotinate. Interestingly, the sulfonate moiety can substitute effectively for the carboxyl group since benzenesulfonate and benzoate displayed similar potency towards the Lac carrier of human neutrophils. This pattern stands in stark contrast to that for the ability of aromatic acids to inhibit Na^+-dependent lactate transport in the kidney [3, 39]. In that preparation, for heterocyclic compounds with a single ring structure, nicotinate and pyrazinoate exert strong inhibition, while picolinate and isonicotinate elicit only mild–moderate inhibition. Introduction of a second ring, as in 1- or 2-naphthoate, leads to a marked decrement in potency. Also, the free carboxylate group is absolutely essential since benzenesulfonate lacks efficacy.

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