Lactic Acid Secretion by Human Neutrophils

Evidence for an H^+ + Lactate$^-$ Cotransport System

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ABSTRACT The pathway by which L-lactate (Lac) crosses the plasma membrane of isolated human neutrophils was investigated. The influx of [^{14}C]Lac from a 2 mM Lac, 145 mM Cl^-, 5.6 mM glucose medium was ~1.5 meq/liter of cell water·min and was sensitive to the organomercurial agent mersalyl (apparent K_i ~20 μM), to α-cyano-4-hydroxycinnamate (CHC), the classical inhibitor of monocarboxylate transport in mitochondria, and to UK-5099 (apparent K_i ~40 μM), a more potent analogue of CHC. Transport was also strongly blocked (>80%) by 1 mM of either 3,5-diiodosalicylic acid, MK-473 (an indanyloxyacetate derivative), or diphenylamine-2-carboxylate, and by 0.4 mM pentachlorophenol, but not by 1 mM ethacrynic acid, furosemide, or the disulfonic stilbenes SITS or H_2DIDS. One-way [^{14}C]Lac efflux from steady-state cells amounted to ~6 meq/liter·min and was likewise affected by the agents listed above. Influx, which was membrane potential insensitive and Na^+ independent, displayed a strong pH dependence: extracellular acidification enhanced uptake while alkalinization inhibited the process (pK′ ~5.7 at 2 mM external Lac). The rate of [^{14}C]Lac influx was a saturable function of external Lac, the K_m being ~7 mM. Steady-state cells exhibited an intracellular Lac content of ~5 mM and secreted lactic acid into the bathing medium at a rate of ~4 meq/liter·min. Secretion was completely suppressed by 1 mM mersalyl which inactivates the carrier, leading to an internal accumulation of Lac. That the Lac carrier truly mediates an H^+ + Lac^- cotransport (or formally equivalent Lac^-/OH^- exchange) was documented by pH-stat techniques wherein an alkalinization of poorly buffered medium could be detected upon the addition of Lac; these pH changes were sensitive to mersalyl. Thus, the Lac carrier of neutrophils possesses several features in common with other monocarboxylate transport systems in erythrocytes and epithelia.

INTRODUCTION

The importance of a route for the egress of lactic acid, the end-product of anaerobic glycolysis, from the cytosol to cell homeostasis has long been recognized. Elucidation

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of the actual pathway(s) by which lactic and other organic acids enter and leave cells was no doubt hampered by the once widely held impression that physicochemical means (i.e., nonionic diffusion) would suffice to satisfactorily account for the movements of a number of organic compounds across biological membranes. Only over the past two decades have specialized membrane transport systems been identified in diverse cell types.

In a series of original articles, Halestrap and Denton (1974, 1975) and Halestrap (1975, 1976) first proposed the existence of a specific monocarboxylate carrier in mitochondrial and erythrocyte membranes on the basis of inhibition of transport by the aromatic substrate analogue α-cyano-4-hydroxycinnamate (CHC). Since these initial reports, their observations have been amply confirmed and the presence of similar transport systems firmly established in a number of different cell types (Spencer and Lehninger, 1976; Deuticke, 1977; Andersen, Tarpley, and Regen, 1978; Ullrich, Rumrich, and Klöss, 1982; Fafournoux, Demigne, and Remesey, 1985; Siebens and Boron, 1987; Trosper and Philipson, 1987). Over the past decade, a considerable body of information has accumulated on the substrate specificity, inhibitor profile, pH dependence, and kinetics of lactate (Lac) transport.

Two distinct classes of monocarboxylate carriers have been described: (*a*) an Na^+ + Lac^- cotransport in kidney and intestine (Hildmann, Storelli, Haase, Barac-Nieto, and Murer, 1980; Mengual, Leblanc, and Sudaka, 1983; Murer and Burckhardt, 1983), and (*b*) an H^+ + Lac^- cotransport found in the kidney, erythrocytes, heart muscle, and Ehrlich ascites tumor cells (Spencer and Lehninger, 1976; Deuticke, 1982; Siebens and Boron, 1987; Trosper and Philipson, 1987). (*a*) In mammalian kidney, an electrogenic Na^+ + Lac^- cotransport (coupling ratio ~2:1) has been localized to the luminal surface (Wright, 1985), whereas in the amphibian, Siebens and Boron (1987) have reported the transporter to be electroneutral. This carrier exhibits broad specificity and is capable of handling a wide variety of organic anions. (*b*) Likewise, the electroneutral H^+ + Lac^- cotransporter in epithelial and nonepithelial cells accepts a number of different substituted monocarboxylates, exhibits a 1:1 stoichiometry, and is reversibly and competitively blocked by CHC. These carriers possess relatively high affinity for physiologically relevant hydroxy- and oxo-substituted fatty acids such as Lac, pyruvate, 3-hydroxybutyrate, acetoacetate, glycerate, and glyoxalate (Deuticke, 1982). Unlike the classical Cl^-/HCO_3^- exchange system of red blood cells, monocarboxylate carriers possess a rather low affinity for inorganic anions such as Cl^- (Halestrap, 1976; Deuticke, 1982; Deuticke, Beyer, and Forst, 1982).

Prompted by the active interest of our laboratory in anion exchange and intracellular pH (pH_i) regulation in human neutrophils, we sought to define the properties of a specific carrier-mediated transport mechanism for lactic acid in these cells. During the course of our work on Cl^-/HCO_3^- exchange, it became apparent that Lac is not transported via the principal anion exchanger of neutrophils (see Discussion). Since these cells which have few mitochondria (Bainton, Ullyot, and Farquhar, 1971) are virtually completely dependent on anaerobic glycolysis for the generation of ATP as a metabolic energy source, lactic acid constitutes the exclusive end-product of glucose metabolism (Hochachka and Mommsen, 1983). Indeed, copious quantities of

lactic acid are produced and can be readily found in the medium bathing these cells (van Zwieten, Wever, Hamers, Weening, and Roos, 1981; Borregaard, Schwartz, and Tauber, 1984). By analogy to erythrocytes as well as renal and intestinal epithelia where specific carriers that handle Lac have been found, we reasoned that a similar mechanism was likely to occur in neutrophils.

Our interest was further enhanced by a recently postulated role for this carrier in maintaining steady-state pH_i in salamander kidney proximal tubules (Siebens and Boron, 1987) since in neutrophils, as in other cells, it is evident that pH-regulatory mechanisms other than Na^+/H^+ exchange and Cl^-/HCO_3^- exchange must be invoked to explain the factors controlling steady-state pH_i (Simchowitz and Cragoe, 1987). While it is true that the extrusion of H^+ equivalents that were originally generated inside the cells (e.g., lactic acid derived from glycolysis) has no material consequence for pH_i, it is quite conceivable that such a pathway could serve as an ancillary mechanism for the efflux of other forms of acid (e.g., H^+ equivalents produced during metabolism and passive H^+, OH^-, or HCO_3^- leaks). All that is required would be to simply couple H^+ movements to those of another transportable anion via the carrier.

As anticipated, we find that isolated human neutrophils do indeed possess a specific transport system for handling L-Lac/lactic acid. The overwhelmingly predominant mechanism seems to be a membrane potential–resistant, Na^+-independent $H^+ + Lac^-$ cotransport that is sensitive to the organomercurial mersalyl and to the aromatic monocarboxylate analogue CHC and other related derivatives. Nonionic diffusion of the undissociated lactic acid molecule (pK′ 3.8) plays little, if any, role. Studies designed to assess substrate selectivity demonstrate broad acceptance of a variety of substituted short-chain aliphatic monocarboxylates as well as aromatic monocarboxylates, but rather low affinity for inorganic anions such as Cl^- and SO_4^{2-}. In general, these properties are strongly reminiscent of those of other previously described monocarboxylate carriers in erythrocytes and epithelia.

MATERIALS AND METHODS

Incubation Media

The standard medium used in this study had the following composition: 137 mM NaCl, 5 mM KCl, 2 mM Na L-lac, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 5 mM HEPES buffer, pH 7.40, and 1 mg/ml of crystalline bovine serum albumin. An all-SO_4^{2-} medium was prepared by substituting SO_4^{2-} salts for those with Cl^-. This medium contained 100 mM SO_4^{2-}, 194 mM Na^+, and 5 mM K^+ in addition to the normal amounts of Ca^{2+} (1 mM), Mg^{2+} (0.5 mM), and HEPES (5 mM). To test the effects of varying external Cl^-, glucuronate and SO_4^{2-} were used as replacement anions. The cation composition of the media was manipulated by substituting equimolar amounts of either K^+ or *N*-methyl-D-glucamine for Na^+. To preserve isotonicity, all of the media were adjusted to a total osmolarity of 305 ± 2 mosmol/liter, as monitored by a vapor pressure osmometer (model 5500; Wescor Inc., Logan, UT). For experiments in which the extracellular pH (pH_o) of the media was varied between 5.0 and 8.3, the solutions were buffered with 2-(*N*-morpholino)ethanesulfonic acid (MES; pK′ 6.0), HEPES (pK′ 7.3), or *N*-Tris(hydroxymethyl)methylglycine (Tricine; pK′ 7.8) as appropriate.

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 (Boyum, 1968). Contaminating erythrocytes were removed by hypotonic lysis in distilled water for 20 s. 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 Lac, 145 mM Cl^-) for 1 h at 37°C before experimentation. All assays were carried out at 37°C.

Reagents and Chemicals

All inorganic salts were obtained from Fisher Scientific Co., St. Louis, MO. The following reagents were purchased from Sigma Chemical Co., St. Louis, MO: crystalline bovine serum albumin, HEPES, MES, *N*-ethylmaleimide (NEM), Tricine, L-lactic acid, sodium lactate, sodium glucuronate, D-glucuronic acid, D-glucose, 2-deoxy-D-glucose (2-DOG), ethacrynic acid, 3,5-diiodosalicylic acid (DISA), mersalyl, pentachlorophenol, probenecid, sodium pyruvate, sodium oxamate, D-lactic acid, sodium isethionate, sodium succinate, sodium citrate, 3-chloropropionic acid, 2-chloroacetic acid, 3-hydroxybutyric acid, 2-chlorobenzoic acid, and 4-hydroxybenzoic acid. CHC was bought from Aldrich Chemical Co., Milwaukee, WI; sodium 4,4′-diisothiodihydrocyanostilbene-2,2′-disulfonate (H_2DIDS) and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) from Pierce Chemical Co., Rockford, IL; diphenylamine-2-carboxylic acid (DPC) from Fluka Chemical Corp., Ronkonkoma, NY; and nigericin from Calbiochem Corp., La Jolla, CA. Amiloride was a generous gift of Dr. Edward J. Cragoe, Jr. of the Merck Sharp & Dohme Research Laboratories, West Point, PA; α-cyano-β-(1-phenylindol-3-yl)acrylic acid (UK-5099) was graciously provided by Pfizer Central Research Laboratories, Sandwich, Kent, UK; and furosemide was kindly supplied by Hoechst-Roussel Laboratories, Somerville, NJ. L-[^{14}C]Lactic acid was purchased from New England Nuclear, Boston, MA; the specific activity was 90 mCi/mmol.

One-Way [^{14}C]Lac 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]Lac (3.0 μCi/ml). At stated intervals, triplicate aliquots of the cell suspensions were layered on 0.5 ml of silicone oil (Versilube F-50; General Electric Corp., Waterford, NY) contained in 1.5-ml plastic tubes and centrifuged for 1 min at 8,000 *g* in a microcentrifuge (Beckman Instruments, Carlsbad, 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 Instrument Co., Inc., Meriden, CT). For efflux studies, neutrophils were loaded with [^{14}C]Lac (6.0 μCi/ml) for 15 min at 37°C in the standard 2 mM Lac medium. Thereafter, because of rapid efflux kinetics, the cells were spun down and either resuspended in the standard Cl^- medium with drugs or else quickly washed once with unlabeled glucuronate medium at 4°C and then resuspended in the various experimental solutions at 37°C. Samples were taken at 5-s intervals through 40 s for measurement of the amount of residual radioactivity that remained associated with the cell pellet. At the stated times, aliquots of the neutrophil suspensions were pipetted into tubes containing an equal volume of ice-cold medium supplemented with mersalyl (final concentration = 0.25 mM, a saturating dose) to abolish all further transport. Samples were then spun over silicone oil and handled as described for influx studies.

Lac Assays

At different times the reactions were terminated by the addition of 10 mM NaF and 1 mM mersalyl, which together stop all further lactic acid production and secretion (Sigma, 1976). Aliquots of the neutrophil suspensions were overlaid on silicone oil and the cells were then pelleted through the oil phase as described above. For secretion studies, the supernatants were saved for assay. In addition, the neutrophils' intracellular Lac content was determined from the cellular pellet after lysis and dispersion in 8% perchloric acid in water. Lac was assayed spectrophotometrically from the change in absorbance of NAD at 340 nm in the presence of excess lactic dehydrogenase according to well-established procedures (Sigma, 1976). Appropriate standards were included daily to assure accuracy. The amount of Lac could then be obtained by comparing optical density readings to the standard curves. Internal Lac concentrations were calculated using an intracellular water volume of 0.274 pl/cell (Simchowitz and Roos, 1985).

pH-Stat Methods

H^+ movements across the cells were monitored from changes in the pH of the bathing solutions using conventional pH-stat techniques. For these experiments, the buffering power of the medium was reduced by lowering the concentration of MES from 5 to 0.1 mM. The neutrophils ($6–8 \times 10^6$/ml) were exposed to the different incubation conditions 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 microliter 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 milliequivalents per 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 pl/cell (Simchowitz and Roos, 1985).

Data Analysis

In most cases, the influx of [^{14}C]Lac, corrected for zero time "uptake," which represents label trapped in the extracellular space, followed equations of the form:

$$C_t = C_\infty[1 - \exp(-kt)] \quad (1)$$

where C_t is the cell label at time t, C_∞ is the cell label at steady state, and k is the rate coefficient. Eq. 1 was fit to the data by a nonlinear least-squares program, and the initial influx rate was computed from the product kC_∞. As indicated in the figure legends, the change in some of the measured variables often appeared to be linear over the period of study; in those cases, the influx rate was computed from the slope of the linear regression line.

The efflux rate coefficients were computed by least-squares fitting the time course data to a single exponential equation. For calculation of initial efflux rates, the starting intracellular Lac concentration at zero time was taken as 5.2 meq/liter of cell water (Fig. 9).

[^{14}C]Lac Background Leak

From the time course of [^{14}C]Lac influx (Fig. 1), it appears that total influx can be divided into two components: one displaying single exponential kinetics (later shown to be mediated through the Lac carrier), and the other a linear slope due to a separate process. The existence of this small second component could also be appreciated in studies with the inhibitor mersalyl. As the drug concentration was raised to 500 or 1,000 μM, levels that exceed the apparent K_i

value (~20 μM) by factors of ~25–50, uptake via the Lac carrier should be negligible. Under these conditions, however, it was evident that influx approached a nonzero value of ~0.036 meq/liter·min at 2 mM Lac. Our initial impression was that this residual flux represented nonionic diffusion of the uncharged form of lactic acid. While this flux was indeed nonsaturable with respect to external Lac, its magnitude remained constant as pH_o was lowered from 7.40 to

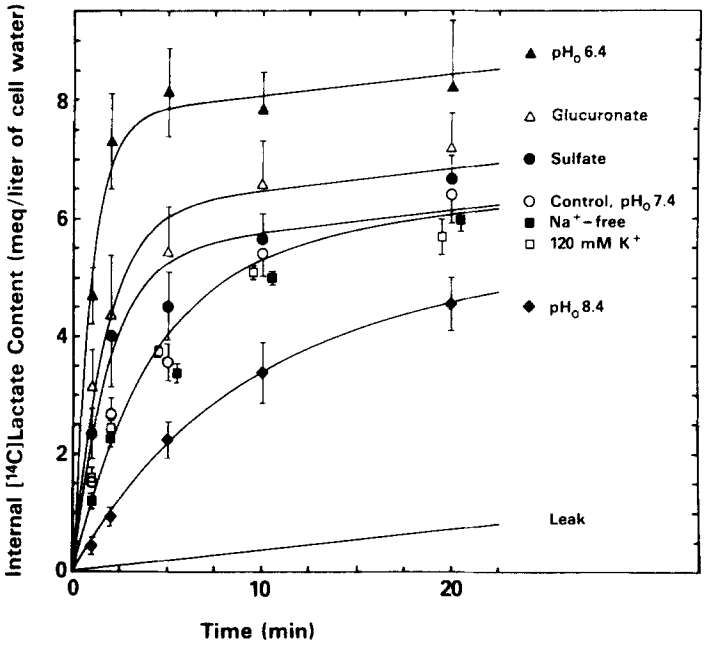

FIGURE 1. Time course of steady-state [^{14}C]Lac influx into human neutrophils: effect of ionic environment. At zero time, aliquots of a neutrophil suspension were resuspended in the standard 2 mM Lac (labeled with 3 μCi/ml [^{14}C]Lac), 145 mM Cl^- medium, pH_o 7.40, under the following experimental conditions. (*a*) Control: 5 mM K^+, 140 mM Na^+; the curve also utilizes data in Na^+-free medium (replacement by *N*-methyl-D-glucamine) and in 120 mM K^+, 25 mM Na^+ medium. (*b*) Standard medium, pH_o 6.40. and (*c*) Standard medium, pH_o 8.40. Studies were also conducted in Cl^--free media, pH_o 7.40 where either (*d*) SO_4^{2-} or (*e*) glucuronate replaced Cl^- (equiosmolar substitution of sulfate or glucuronate salts for those of Cl^-). At stated times, samples were removed and the cell pellets were isolated and counted for radioactivity. The data points were fit to single exponential equations (Eq. 1) superimposed on a linear slope of 0.036 meq/liter·min (representing some form of pH-insensitive, inhibitor-resistant leak) which yielded initial influx rates (in meq/liter·min) for the exponential component as follows: 1.4 ± 0.2 (*a*), 8.1 ± 1.6 (*b*), 0.54 ± 0.18 (*c*), 3.0 ± 0.6 (*d*), and 3.8 ± 1.2 (*e*). The final uptake values (in meq/liter of cell water) for the exponentials were: 5.4 ± 0.2 (*a*), 7.7 ± 0.5 (*b*), 4.2 ± 0.6 (*c*), 5.4 ± 0.4 (*d*), and 6.1 ± 0.5 (*e*). Results have been taken from three to five separate experiments for each condition.

6.40, in marked contrast to the expected 10-fold increase if this flux were truly taking place via nonionic diffusion.

Two possibilities exist as to the nature of the slow component, which appears to reflect continuous uptake of radioactivity after the establishment of the steady state (Fig. 1): (*a*) intracellular metabolic conversion of [^{14}C]Lac, or (*b*) parallel uptake of a labeled impurity

chemically different from L-Lac. The first alternative is unlikely since pretreatment of cells with the metabolic inhibitor 2-deoxy-D-glucose failed to alter the slow component of "influx." In all probability, the background (or so-called leak) does not actually represent true uptake, but rather increased trapping of label in the extracellular fluid due to aggregation of cells over time. Two lines of evidence serve to corroborate this notion: (*a*) use of Ca^{2+}- and Mg^{2+}-free medium to prevent cell adherence markedly reduces the magnitude of the residual flux, and (*b*) increased trapping of extracellular fluid could be detected using [^{14}C]inulin. Conceivably, uptake of a labeled trace contaminant of L-Lac could also play a role. Suffice it to say, however, that the weight of evidence suggests that this background uptake results from technical factors and is unrelated to any transport pathway involving Lac. Therefore, we have considered this very minor fraction ($\sim$3% of the total influx rate under steady-state conditions) to be a background and subtracted this from the measured isotopic uptake in calculating [^{14}C]Lac fluxes through the carrier in all figures and tables appearing after Fig. 1.

RESULTS

[^{14}C]Lac Fluxes

The time course of [^{14}C]Lac influx from a 2 mM Lac medium is presented in Fig. 1. In the normal 145 mM Cl^- medium (control), uptake followed single exponential kinetics superimposed on a linear slope. (As indicated above, this latter flux was nonsaturable and inhibitor resistant, strongly suggesting that it represents some form of background. Moreover, the flux was not altered by lowering pH_o from 7.40 to 6.40, thereby implying that it could not be ascribed to nonionic diffusion of the uncharged form of lactic acid.) The exponential component for control conditions (5 mM K^+, 140 mM Na^+) displayed an initial influx rate of 1.4 ± 0.3 meq/liter of cell water·min. The final extrapolated uptake value of 5.6 ± 0.4 meq/liter of cell water signifies the level at which isotopic equilibrium has taken place and provides an estimate of the internal Lac content ($[Lac^-]_i$) of steady-state neutrophils under these conditions. (As will be seen below, this value agrees well with that for intracellular Lac determined chemically by means of a spectrophotometric assay.) This time course was not appreciably altered by external Na^+ removal (Fig. 1), equimolar replacement by *N*-methyl-D-glucamine). Moreover, depolarization of the membrane potential from its normal resting value of ~ -55 to ~ 0 mV (Seligmann and Gallin, 1980; Simchowitz, Spilberg, and De Weer, 1982), achieved by raising external K^+ from 5 to 120 mM, caused little alteration in the influx rate (Fig. 1).

Fig. 1 also displays data on the effect of external Cl^- removal where all Cl^- (145 mM) in the bathing solutions was replaced by either glucuronate (145 mM) or SO_4^{2-} (100 mM, to maintain isotonicity). Both of these substitutions were associated with a significant enhancement in the rate of [^{14}C]Lac influx (3.8 ± 1.2 and 3.0 ± 0.6 meq/liter·min, respectively) relative to Cl^-, the increase being somewhat greater in glucuronate medium. The final steady-state uptake values were roughly the same for all three treatment conditions (Cl^-, SO_4^{2-}, and glucuronate) however: 5.6 ± 0.4, 5.4 ± 0.4, and 6.1 ± 0.5 meq/liter of cell water, at least within experimental error. Since only the rate, but not the final extent of uptake, seemed to be altered by the presence of the different extracellular anions, these findings suggest the possibility that Cl^- and to a much lesser extent SO_4^{2-} may be competing with Lac at the external translocation site of a putative carrier for which glucuronate exhibits the least affinity. The results of Fig. 1 also demonstrate the marked sensitivity of this presumptive

transport to pH: extracellular acidification to pH_o 6.40 stimulates the initial rate of [^{14}C]Lac influx approximately sixfold, while alkalinization to pH_o 8.40 suppresses influx by a factor of ~3.

Data on the inhibitory profile of different drugs are presented in Fig. 2. As compared with control conditions in the standard 2 mM Lac, 145 mM Cl^- medium at pH_o 7.40 (influx rate = 1.5 ± 0.3 meq/liter·min), the time course of [^{14}C]Lac influx was not appreciably affected by 0.1 mM ouabain (a Na^+/K^+ pump inhibitor), 1 mM

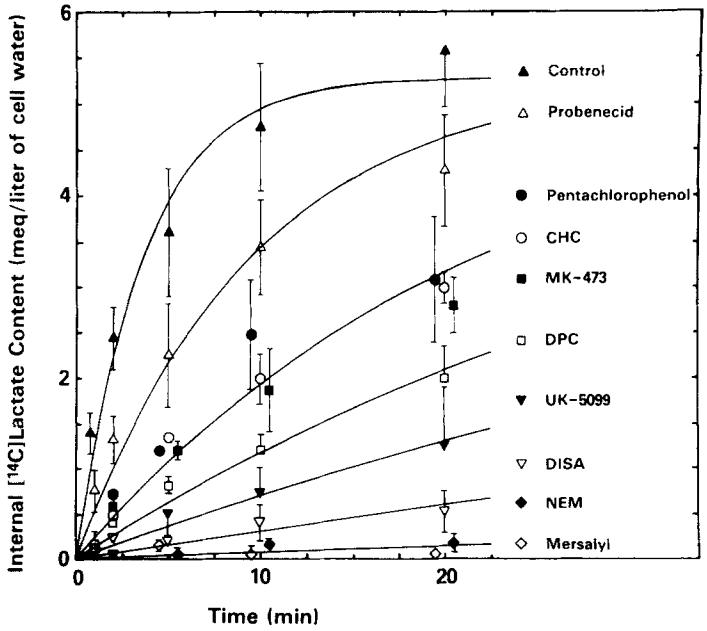

FIGURE 2. Time course of steady-state [^{14}C]Lac influx into human neutrophils: effect of drugs. See legend to Fig. 1. Control: standard 2 mM Lac, 145 mM Cl^- medium, pH_o 7.40, in the presence of either 10 mM CHC, 1 mM MK-473, 1 mM DPC, 0.4 mM pentachlorophenol, 0.4 mM UK-5099, 1 mM mersalyl, 1 mM DISA, 1 mM NEM, or 1 mM probenecid. Each of the drugs was evaluated in medium containing 5 mM K^+ and 140 mM Na^+. The data points were fit to a single exponential (after subtraction of a linear, background slope of 0.036 meq/liter·min) which gave initial influx rates (in meq/liter·min) of: 1.5 ± 0.3 (control, where final uptake = 5.3 ± 0.5 meq/liter of cell water), 0.56 ± 0.09 (probenecid), 0.24 ± 0.02 (combined data for pentachlorophenol, CHC, and MK-473), 0.13 ± 0.01 (DPC), 0.074 ± 0.019 (UK-5099), 0.032 ± 0.010 (DISA), and 0.007 ± 0.003 (combined data for NEM and mersalyl). Results represent three to four experiments for each drug.

amiloride (a Na^+/H^+ exchange inhibitor), or 1 mM of either furosemide, ethacrynic acid, SITS, or H_2DIDS (commonly used inhibitors of diverse transport systems). Probenecid (1 mM), which blocks organic acid transport in several epithelial tissues (Blomstedt and Aronson, 1980), exhibited moderate suppression (62%) in neutrophils. The compound CHC, the classical inhibitor of monocarboxylate transport in mitochondria and other cells (Halestrap and Denton, 1974, 1975), caused a higher degree of inhibition (84%), but required a concentration of 10 mM to achieve these

suppressive effects. On the other hand, 0.4 mM pentachlorophenol, 1 mM MK-473 (an indanyloxyacetate derivative), 1 mM diphenylamine-2-carboxylate, 0.4 mM UK-5099 (a cinnamate analogue), 1 mM DISA, 1 mM NEM, or 1 mM mersalyl all markedly suppressed influx with initial rates of 0.27 ± 0.05, 0.22 ± 0.02, 0.13 ± 0.01, 0.07 ± 0.02, 0.03 ± 0.01, 0.010 ± 0.005, and 0.0045 ± 0.0041 meq/liter·min, respectively. These values correspond to inhibitions of 82, 85, 91, 95, 98, 99, and 99%. It should be noted at the outset that all of these agents are weak organic acids by virtue of their carboxyl or phenolic groups, which presumably exhibit pK′ values in the range of 4–6. However, as stated above, other anionic compounds such as furosemide, ethacrynate, and SITS have little or no activity in this system. One would anticipate any potential uptake of [^{14}C]lactic acid via nonionic diffusion to be generally resistant to these maneuvers. Therefore, suppression of [^{14}C]Lac influx by a variety of agents tends to favor a carrier-mediated mechanism.

Data on the efflux of [^{14}C]Lac are presented in Fig. 3. The efflux from steady-state neutrophils bathed in the standard Cl^- medium containing 2 mM Lac was first-order with a rate coefficient of 1.21 ± 0.04 min^{-1}. Taking the resting internal Lac content to be 5.2 meq/liter of cell water (the value measured chemically; see Fig. 9), the rate coefficient signifies an initial [^{14}C]Lac efflux rate of 6.3 ± 0.2 meq/liter·min, roughly fourfold greater than for one-way influx (Figs. 1 and 2). Efflux was markedly reduced in the presence of 0.4 mM pentachlorophenol, 0.4 mM UK-5099, 1 mM DISA, and 0.25 mM mersalyl, the degrees of inhibition (78, 88, 93, and 98%, respectively) being comparable to those observed for the influx experiments of Fig. 2. Similar results were noted with 1 mM of either DPC or MK-473 in that one-way [^{14}C]Lac influx and efflux were likewise affected.

Further evidence in support of carrier-mediated transport is provided by the data of Fig. 4, which displays the dose–response curves for [^{14}C]Lac influx as a function of extracellular Lac (0.16–20 mM). Experiments were conducted in Cl^- as well as in glucuronate medium. In both cases, saturation is clearly evident with apparent K_m values for Lac of 7.0 ± 1.8 and 13 ± 4 mM in glucuronate and Cl^- media, respectively. However, the parameters for V_{max} were statistically indistinguishable (9.2 ± 1.0 vs. 8.8 ± 1.5 meq/liter·min), consistent with the idea that Cl^- possesses some affinity for the carrier and thus behaves as a competitive inhibitor of Lac while glucuronate does not (or at least exhibits considerably less affinity than does Cl^-). The fact that the uptake of [^{14}C]Lac demonstrates saturation kinetics provides strong confirmatory evidence that a carrier-mediated transport system exists and that nonionic diffusion probably makes little or no contribution to the total flux. Moreover, on the assumption that glucuronate is inert with respect to the Lac carrier, it follows from simple competition kinetics that an approximately twofold increase in K_m (Lac^-) from 7.0 to 13 mM in going from 145 mM glucuronate to Cl^- medium implies a K_m for Cl^- that is ~145 mM. We shall return to this point later.

The effect of extracellular pH on the influx of [^{14}C]Lac is shown in Fig. 5 *A*. Extracellular acidification stimulated influx while extracellular alkalinization inhibited it. The relationship between influx and pH_o could be adequately described by a titration curve with a pK′ of 5.7 ± 0.1. If uptake were taking place exclusively via nonionic diffusion of the neutral (protonated or uncharged), free acid form, one would have predicted a pK′ of ~3.8, the dissociation constant for lactic acid. The

disparity between this and the observed value emphasizes once again that nonionic diffusion seems to play little role in the transport of Lac into the cells.

A plausible explanation for the pH dependence curve is that, while the carrier may actually be operating to take up lactic acid, the underlying mechanism is probably that of an $H^+ + Lac^-$ cotransport, in which case protons serve as a substrate. Alternatively, H^+ could be exerting some allosteric effect to increase the maximal transport rate of the reaction. Some evidence against the latter possibility is provided

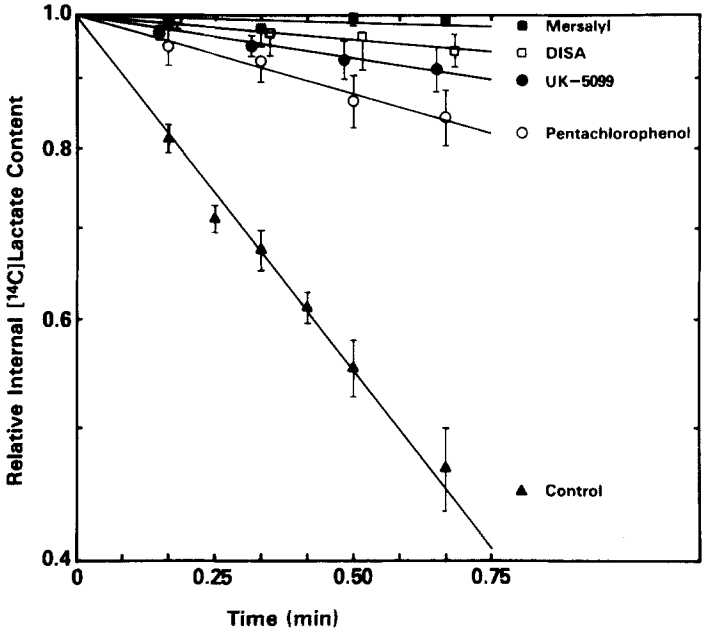

FIGURE 3. Time course of [^{14}C]Lac efflux and the effect of several drugs. Neutrophils were loaded with [^{14}C]Lac for 15 min at 37°C in the standard 2 mM Lac medium. Thereafter, because of very rapid efflux kinetics, the cells were spun down and resuspended in unlabeled medium containing 2 mM Lac with or without drugs. At stated times the cell pellets were isolated and counted for radioactivity. Efflux rate coefficients were obtained by least-squares fitting the data points to a declining single exponential equation. Taking the intracellular Lac content of these steady-state neutrophils to be 5.2 meq/liter of cell water (see Fig. 9), the initial [^{14}C]Lac efflux rates (in meq/liter·min) were as follows: 6.3 ± 0.2 (control), 1.4 ± 0.2 (in the presence of 0.4 mM pentachlorophenol), 0.77 ± 0.16 (0.4 mM UK-5099), 0.44 ± 0.13 (1 mM DISA), and 0.14 ± 0.11 (0.25 mM mersalyl). Results have been taken from three to four experiments.

by the data of Fig. 5 *B*, which displays the effect of pH_i on [^{14}C]Lac influx. For these experiments, the pH_i was manipulated at constant pH_o (7.40) by varying extracellular K^+ in the presence of 2 μM nigericin, a K^+/H^+ exchanging ionophore (Pressman, 1969), as previously described (Simchowitz and Davis, 1989). As shown, in contrast to the dramatic effects of pH_o on influx rate, changes in the pH_i between 6.2 and 7.4 were relatively modest and in the opposite direction, acidification reducing influx. These findings suggest that H^+ might need to be on the same (*cis*) side of the plasma

membrane as Lac^- to exert its effect. Such an interpretation is in keeping with the proposed model of an H^+ + Lac^- cotransport mechanism alluded to above. However, the possibility still exists that the nature of any hypothetical pH-regulatory sites of carrier activity could be different on the inward- and outward-facing states.

In association with the substantial effects of pH_o on the velocity of transport, the data of Fig. 6 indicate that the affinity of the carrier for Lac also varies to some degree. As shown, at pH_o 5.30, 6.30, and 7.40, the K_m values from Lineweaver-Burk plots for external Lac were 4.9 ± 1.1, 5.4 ± 1.3, and 13 ± 4 mM, respectively.

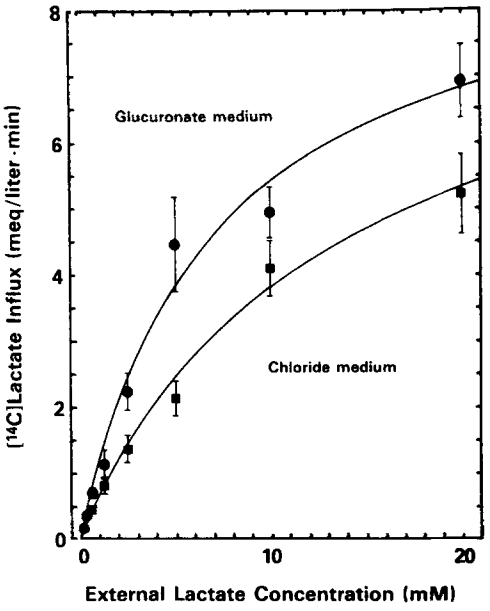

FIGURE 4. Substrate saturation: rate of [^{14}C]Lac influx as a function of external Lac^- in either Cl^- or glucuronate medium. Neutrophils were bathed in either 5 mM K^+, Cl^- medium or 85 mM K^+, glucuronate medium, also containing 2 μM nigericin, to avoid any intracellular alkalinization that would otherwise occur through reverse Cl^-/HCO_3^- exchange in this Cl^--free solution. In the presence of 85 mM K^+ and nigericin, the cells are effectively pH-clamped at their normal resting pH_i of ~7.25 (Simchowitz and Roos, 1985). The internal [^{14}C]Lac contents were measured at 1 and 2 min and the initial [^{14}C]Lac influx rates were calculated as in Fig. 1 by fitting the data points to single exponential equations (Eq. 1). A linear flux = 0.018 $(Lac^-)_o$/min (presumably representing some inhibitor-resistant, nonsaturable leak) was considered as carrier-independent background and subtracted in deriving the initial rates through the Lac transporter. The initial influx rates were then plotted against the prevailing extracellular Lac concentration of the bathing medium (0.16–20 mM). Both curves show substrate saturation by external Lac. Fits to a Michaelis-Menten activation equation yielded the following kinetic parameters: $K_m(Lac^-)$ = 7.0 ± 1.8 and 13 ± 4 mM and V_{max} = 9.2 ± 1.0 and 8.8 ± 1.5 meq/liter·min in glucuronate and Cl^- media, respectively. Results are from three to four experiments.

Another linear transformation of the same data, namely, a Hanes-Woolf plot ([S]/v vs. [S]; not shown) yielded K_m values that were similar to those above: 5.5 ± 1.3, 6.2 ± 1.4, and 15 ± 2 mM, respectively.

Data on the dose dependence of several inhibitors are shown in Fig. 7. At 2 mM Lac, mersalyl, UK-5099, and DISA each reduced the influx rate of [^{14}C]Lac along a Michaelis-Menten inhibition equation that yielded apparent K_i values of 11 ± 3, 41 ± 10, and 135 ± 29 μM, respectively.

The reversibility of the various drugs was next examined by pretreating the cells with the various inhibitors for 15 min followed by washing the cells and then

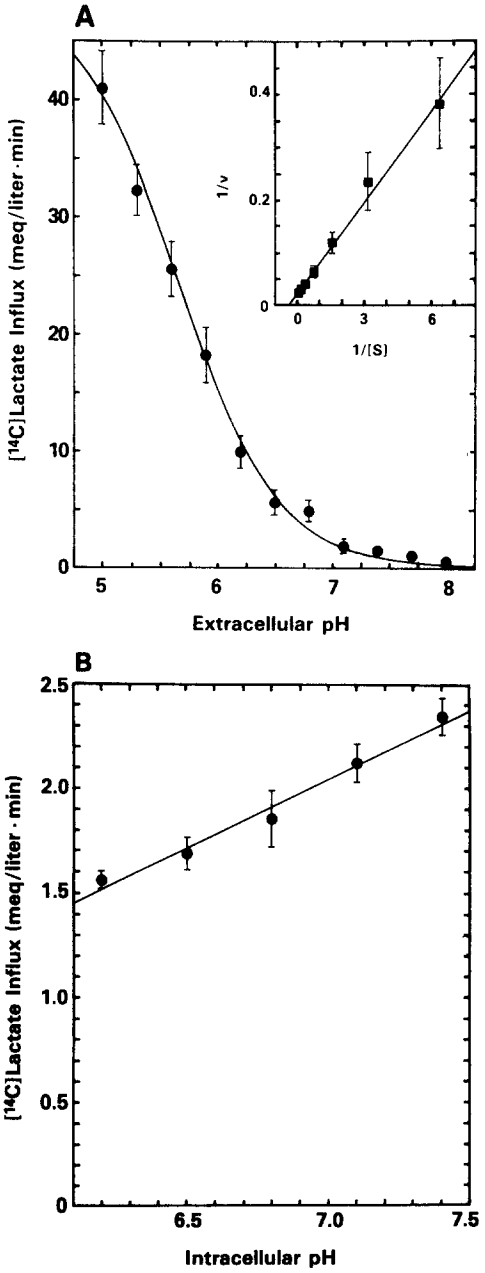

FIGURE 5. (*A*) Dependence of [^{14}C]Lac influx on extracellular pH. One-way [^{14}C]Lac influx was measured as in Fig. 1 from a 2 mM Lac^-, 145 mM Cl^- medium in which the pH_o was varied between 5.0 and 8.0. Results are from four separate experiments. The data points were fit to a titration curve by a nonlinear least-squares program. The inflection point, corresponding to an apparent pK, is at 5.7 ± 0.1 ($K_m(H^+)$ = 2.0 ± 0.4 μM). The inset displays the data in double-reciprocal fashion (Lineweaver-Burk plot), where [S] = $[H^+]_o$ (micromolar). (*B*) Dependence of the rate of [^{14}C]Lac influx on intracellular pH. The neutrophils were resuspended in 2 mM Lac, 145 Cl^- medium, pH_o 7.40, also containing 2 μM nigericin, in which the extracellular K^+ concentration was varied between 7.5 and 120 mM by replacement of Na^+. Under these conditions, $[K^+]_o/[K^+]_i = [H^+]_o/[H^+]_i$ and since the resting $[K^+]_i \sim 120$ meq/liter of cell water (Simchowitz et al., 1982), the $[H^+]_i$ (hence also pH_i) can be calculated from the above equation. Accordingly, at $[K^+]_o$ = 7.5, 15, 30, 60, and 120 mM, the estimated pH_i values are 6.20, 6.50, 6.80, 7.10, and 7.40, respectively. The influx of [^{14}C]Lac into the cells was measured at 1 and 2 min. The initial influx rates were calculated as in Fig. 1 and plotted against the estimated pH_i. The curve through the data points is a straight line, the equation for the linear regression being: rate (in meq/liter·min) = 0.66 pH_o − 2.6. Results represent three separate experiments.

determining the rate of [^{14}C]Lac influx. By these means, it was readily apparent that UK-5099, DISA, pentachlorophenol, diphenylamine-2-carboxylate, and MK-473 were completely reversible, whereas the action of mersalyl was at least partially irreversible.

A number of other weak organic acids were evaluated as potential substrates for the

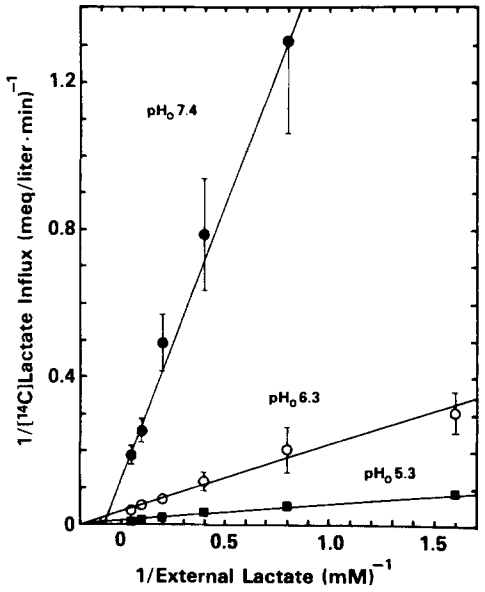

FIGURE 6. Lineweaver-Burk plot of the activation of [^{14}C]Lac influx by extracellular Lac at pH_o 5.3, 6.3, and 7.4. See legend to Fig. 4, except that similar experiments were also performed in Cl^- media without nigericin at pH_o 5.3 and 6.3. The line labeled pH_o 7.4 represents the same data as shown in Fig. 4, but graphed in double-reciprocal fashion, as are the data obtained at pH_o 5.3 and 6.3. Fits to the individual sets of data yielded apparent K_m values for external Lac of 4.9 ± 1.1, 5.4 ± 1.3, and 13 ± 4 mM, and V_{max} values of 105 ± 24, 29 ± 6, and 8.8 ± 1.5 meq/liter·min, respectively, at pH_o 5.3, 6.3, and 7.4.

Lac transporter (Table I). For these experiments, the ability of the test anions to bind to the external translocation site of the carrier and thus to compete with Lac for uptake was examined. The degree of *cis* inhibition of [^{14}C]Lac influx from a 2-mM Lac medium by the compounds was thus a function its affinity. The data of Table I reveal the relative nonselectivity of the Lac carrier as manifested by its broad acceptance of a variety of substituted short-chain aliphatic and aromatic anions. For

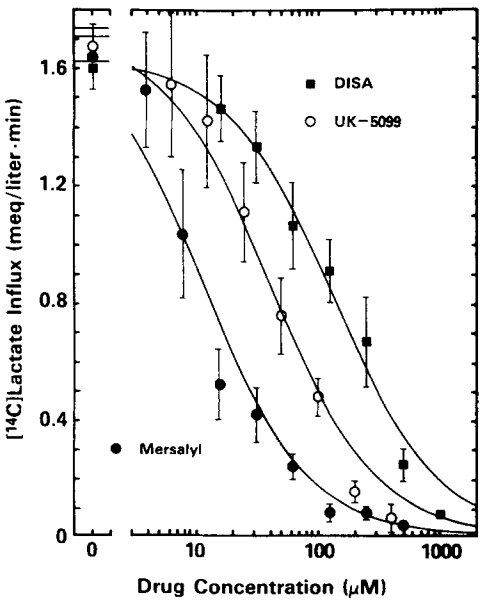

FIGURE 7. Dose dependence of inhibition of [^{14}C]Lac influx by various drugs. The uptake of [^{14}C]Lac into neutrophils from a 2 mM Lac (balance Cl^-) medium was measured in the presence of varying concentrations of either mersalyl (0–500 μM), DISA (0–1,000 μM), or UK-5099 (0–400 μM). The initial [^{14}C]Lac influx rates were calculated as in Figs. 1 and 2 by fitting the internal [^{14}C]Lac contents at different time intervals to single exponentials (Eq. 1). The curves represent Michaelis-Menten inhibition equations which yielded apparent K_i values of 11 ± 3, 41 ± 10, and 135 ± 29 μM for mersalyl, UK-5099, and DISA, respectively. Results are from three to four experiments for each condition.

example, 2-chloroacetate, oxamate, 3-chloropropionate, pyruvate, 2-chlorobenzoate, and 4-hydroxybenzoate all appear to bind to the same external transport site with affinities roughly comparable to that for Lac (apparent K_m in Cl^- medium = 13 ± 4 mM; Fig. 4), the former two actually displaying somewhat higher affinity. It should be stressed that due to similarities in structure, we have assumed inhibition of [^{14}C]Lac influx by these compounds to be indicative of competition, although this point has not as yet been proven in a strict sense. Interestingly, the D- and L-stereoisomers of Lac display quite different activities in this system, the carrier demonstrating a preference for the L- over the D-form by a factor of ~3. Di- and tricarboxylates such as succinate and citrate, as well as some monocarboxylates like isethionate, are devoid of affinity.

TABLE I

Substrate Competition: Kinetic Constants for Inhibition of [^{14}C]Lac Influx by Various Organic Anions

Anion	Apparent K_i
	mM
2-Amino-2-ketoacetate (oxamate)	4.9 ± 0.6
2-Chloroacetate	6.4 ± 1.3
3-Chloropropionate	10 ± 2
2-Ketopropionate (pyruvate)	13 ± 3
2-Chlorobenzoate	14 ± 3
4-Hydroxybenzoate	17 ± 5
3-Hydroxybutyrate	22 ± 8
D-2-hydroxypropionate (D-Lac)	36 ± 13
Isethionate	—*
Succinate	—*
Citrate	—*

The bathing solutions contained 125 mM Cl^- in which the concentrations of the different anions were varied between 0 and 20 mM by replacement of glucuronate. The [^{14}C]Lac influx rates were calculated as in Fig. 1 and plotted against the external concentrations of the competing anions. The data set for each anion was then fit to a Michaelis-Menten inhibition equation which yielded the apparent K_i values listed above. These parameters signify the apparent K_m values for each of the various anions in the presence of 2 mM Lac. Results represent three experiments for each anion.
*No inhibition at 20 mM.

As noted in Figs. 1 and 4, however, it is readily demonstrable that the carrier possesses a finite affinity for Cl^-, a property that may have a direct bearing on the biologic function of this membrane transport system (see Discussion). This point was tested in a more quantitative way by examining the influx of [^{14}C]Lac from a nominally inert glucuronate medium as a function of external Cl^- (data not shown). In this case, Cl^- suppressed [^{14}C]Lac uptake with an apparent K_i of 122 ± 33 mM, which corresponds to the apparent K_m value for Cl^- in the presence of 2 mM Lac. Since glucuronate is presumably devoid of affinity for the carrier, or nearly so, and K_m (Lac) is ~7 mM (Fig. 4), it follows from simple competition kinetics that the true half-saturation constant for Cl^- activation of transport is ~95 mM.

Lactic Acid Secretion: Intracellular Lac

Additional studies were performed on the secretion of lactic acid (Fig. 8), the biologically relevant direction of transport, and correlated with determinations of the internal Lac content of these cells (Fig. 9). Fig. 8 displays the time course of efflux under a variety of experimental conditions. All effluxes were linear and the rate of lactic acid secretion from control cells bathed in 5.6 mM glucose medium was 3.7 ±

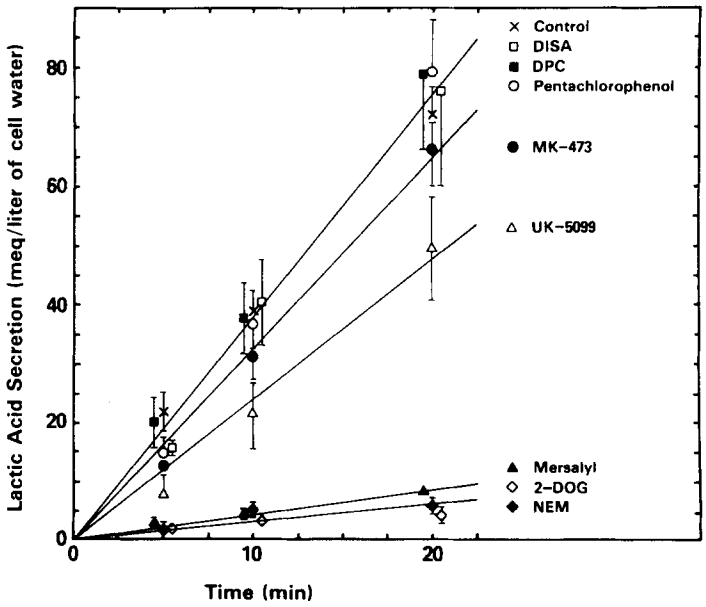

FIGURE 8. Time course of lactic acid secretion from human neutrophils: effect of various drugs. See legends to Fig. 1 and 2, except that 2 mM Lac was omitted from the standard Cl^- medium during the experiment. The appearance of lactic acid in the bathing solution was followed over time from the change in absorbance of NAD at 340 nm using a spectrophotometric assay. Over the time frame of the experiments, all effluxes were linear. The values for the secretion rates (in meq/liter·min) were as follows: 3.8 ± 0.1 (combined data for control, 1 mM DISA, 1 mM DPC, and 0.4 mM pentachlorophenol), 3.2 ± 0.2 (1 mM MK-473), 2.4 ± 0.3 (0.4 mM UK-5099), 0.42 ± 0.04 (1 mM mersalyl), and 0.30 ± 0.03 (combined data for 1 mM NEM and 1 mM 2-DOG). For 2-DOG only, the neutrophils were pretreated with the drug for 15 min. In all other instances, the compounds were added to the cell suspensions at zero time. Results are from three to four experiments for each condition.

0.2 meq/liter·min. The internal Lac concentration of the neutrophils was 5.2 ± 0.5 meq/liter of cell water, a value that remained constant over the entire period of observation (Fig. 9). In agreement with the results of Fig. 1 on [^{14}C]Lac influx, the secretion rate was not significantly affected by removal of extracellular Na^+ or by raising extracellular K^+ to 120 mM, which completely depolarized the membrane potential (data not shown). Likewise, replacement of all extracellular Cl^- by glucuronate or SO_4^{2-} had no appreciable effect on lactic acid secretion (not shown). In

contrast, pretreatment of cells with 1 mM 2-DOG in glucose-free medium led to a marked reduction in efflux, the rate declining from 3.7 to 0.24 ± 0.04 meq/liter·min. This effect is readily understandable in terms of the action of 2-DOG as a metabolic inhibitor that blocks glycolysis and therefore the production of lactic acid. As expected, the $[Lac^-]_i$ of these 2-DOG-treated neutrophils fell in parallel with secretion, the level falling from 5.2 mM to nearly 0 by 20 min. Similar results were seen with 1 mM NEM.

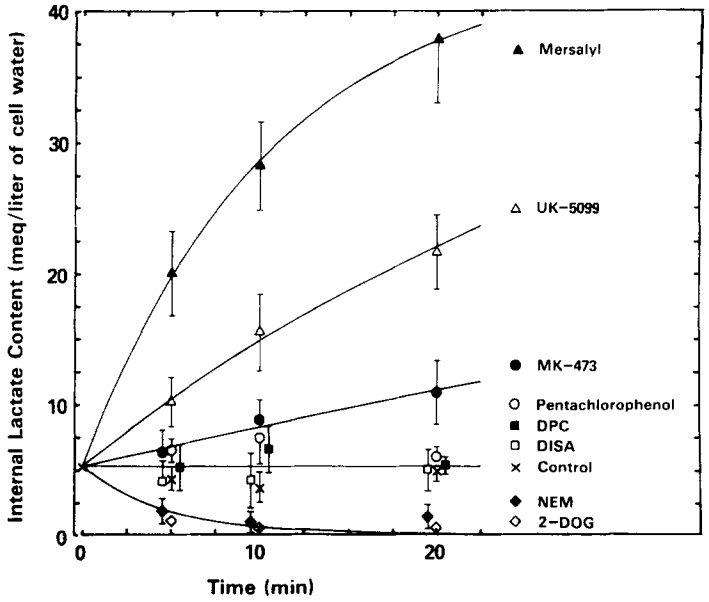

FIGURE 9. The effects of different agents on the intracellular Lac content of human neutrophils. The cells were bathed in the standard 145 mM Cl^- medium containing 5.6 mM glucose, but lacking 2 mM Lac as in Fig. 8. At zero time, either 1 mM mersalyl, 0.4 mM UK-5099, 1 mM DISA, 1 mM MK-473, 1 mM DPC, 0.4 mM pentachlorophenol, 1 mM NEM, or 1 mM 2-DOG was added to the cell suspensions. Glucose was omitted from the medium only when 1 mM 2-DOG was being evaluated. At stated times, the internal Lac concentration of the cells was measured spectrophotometrically. The data points for control, DISA, pentachlorophenol, and DPC were indistinguishable: a horizontal line has been drawn at 5.2 mM, the overall average. The curves for mersalyl, UK-5099, and MK-473, as well as NEM and 2-DOG (combined data) are single exponential fits with initial rates of Lac accumulation of 3.6 ± 0.7, 1.1 ± 0.2, 0.32 ± 0.09, and −1.2 ± 0.2 meq/liter·min, respectively. Results have been taken from three to four separate experiments.

On the other hand, 1 mM mersalyl and 0.4 mM UK-5099 evidently suppress lactic acid secretion (Fig. 8) as well as [^{14}C]Lac influx (Fig. 2). These two drugs in all likelihood inactivate the carrier (or at least strongly inhibit its transport rate) since exposure of neutrophils to these agents was associated with a progressive rise in the internal Lac concentration of the cells (Fig. 9). With mersalyl, $[Lac]_i$ gradually rose with time from its normal resting value of 5.2 meq/liter of cell water to reach a level of ~40 mM by 20 min of incubation. The initial rate of Lac accumulation (3.6 ± 0.7

meq/liter·min) is identical to that for control lactic acid secretion (3.8 ± 0.1 meq/liter·min; Fig. 8), thereby indicating that 1 mM mersalyl causes immediate and essentially complete inhibition of Lac transport. Likewise, in the case of 0.4 mM UK-5099, $[Lac]_i$ progressively increased over time though the rate (1.1 ± 0.2 meq/liter·min) was distinctly less (~30%) than with mersalyl. In contrast, with the possible slight exception of MK-473, neither DISA, MK-473, pentachlorophenol, nor DPC had any effect on lactic acid secretion or on the internal Lac level of the cells (Figs. 8 and 9).

Given the data of Fig. 3 that DISA, pentachlorophenol, and UK-5099 all reduce [^{14}C]Lac efflux by ≥80%, the observations that DISA and pentachlorophenol have no detectable effect on lactic acid secretion while UK-5099 inhibits by only ~35% were rather unexpected. At first, we considered the possibility that this apparent disparity might be due to the presence of 2 mM Lac in the medium during the former but not

TABLE II

Time Course of [^{14}C] Lac Efflux in the Presence of DISA or Mersalyl

Time	Drug	Relative internal [^{14}C]Lac content	
		Actual	Predicted
min			
1	DISA	0.985 ± 0.018	0.919
2.5	DISA	0.408 ± 0.030	0.809
5	DISA	0.043 ± 0.010	0.655
1	Mersalyl	1.003 ± 0.029	0.973
2.5	Mersalyl	1.019 ± 0.031	0.935
5	Mersalyl	0.977 ± 0.023	0.874

Studies were conducted in the presence of 1 mM DISA or 0.25 mM mersalyl under conditions identical to those described in Fig. 3 except that efflux was measured at longer time intervals. Results, which represent the radioactivity associated with the cell pellet at a given time relative to that at zero time, are from four experiments. The column heading "Actual" denotes intracellular contents that were obtained experimentally, whereas the "Predicted" values were calculated based on the mean efflux rate coefficients determined in Fig. 3.

the latter experiments. This, however, proved not to be the case as studies measuring lactic acid secretion and intracellular Lac contents with and without external Lac gave similar results with regard to drug inhibition. As indicated in Table II, the actual explanation, which relates to the marked difference in total incubation times (0.7 min for one-way tracer efflux as opposed to 20 min for the lactic acid secretion determinations), was quite surprising. The data of Table II reveal a striking loss of inhibition of Lac transport after the first minute of incubation despite maintained exposure of the neutrophils to 1 mM DISA. The rapid and complete abrogation of the block of [^{14}C]Lac efflux (within 2–5 min) thus explains why at 10 and 20 min lactic acid secretion is normal in the presence of DISA and why no intracellular accumulation of Lac occurs. The mechanism by which this dramatic reversal of inhibition of the Lac carrier is achieved is unknown, although it would seem that this process must take place at the cytoplasmic surface of the plasma membrane since

[^{14}C]Lac influx remains suppressed at 10–20 min (Fig. 2). Similar results were noted with pentachlorophenol, DPC, MK-473, and to a lesser extent with UK-5099. In contrast, the inhibition of Lac transport by mersalyl was constant over time (Table II).

Dose–response curves were also generated for inhibition of lactic acid secretion by mersalyl and for the build-up of Lac inside the cells (Fig. 10). In the normal Cl^- medium, raising the concentration of mersalyl between 0 and 500 μM led to a progressive reduction in the efflux rate. The data points fell along a Michaelis-Menten inhibition equation which yielded an apparent K_i value of 23 ± 5 μM. This value is similar to that reported for blocking [^{14}C]Lac influx (11 ± 3 μM; Fig. 7) and also to that causing an intracellular accumulation of lactic acid (31 ± 8 μM; Fig. 10).

The effect of extracellular pH on lactic acid secretion is given in Fig. 11. The data indicate that over the pH_o range 5.0–8.3, the relationship is approximately linear

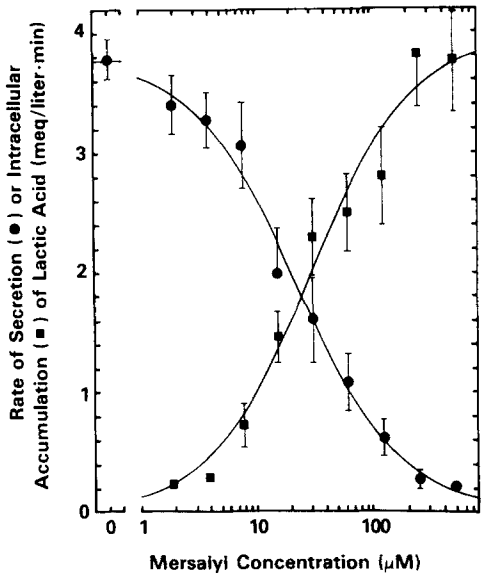

FIGURE 10. Dose dependence of inhibition by mersalyl of lactic acid secretion by human neutrophils. The relationship of the mersalyl concentration to the intracellular accumulation of lactic acid is also shown for comparison. The cells were resuspended in the standard Cl^- medium (but without 2 mM Lac) in the presence of varying concentrations (0–500 μM) of mersalyl. The efflux of lactic acid into the medium and the internal Lactate levels of the cells were measured at 5, 10, and 20 min as described in the legends to Figs. 8 and 9. The data points were fit to Michaelis-Menten inhibition and activation equations which yielded kinetic constants of 23 ± 5 and 31 ± 8 μM, respectively, for secretion and for the build-up of lactic acid. Results are from four to six experiments.

and that the rate of lactic acid efflux is directly proportional to pH_o. Comparable data for the intracellular Lac contents are also provided in Fig. 11. It would appear that $[Lac]_i$ remains roughly constant between pH_o 7.1 and 8.0, but then rises dramatically as the pH_o is progressively lowered toward 5.0.

The fact that secretion (net efflux) of lactic acid into a nominally inert glucuronate medium remains normal (data not shown) implies that the unloaded carrier is capable of readily crossing the membrane and returning to bind Lac at its inward-facing site. Under physiological conditions, the Lac transporter is primarily involved in mediating net Lac efflux. However, since both influx and efflux are measurable, clearly the carrier also operates in an exchange mode. In terms of kinetics, it is of some interest whether the loaded or unloaded transporter crosses the membrane faster; i.e., during the net efflux cycle, does the translocation of Lac or the return of

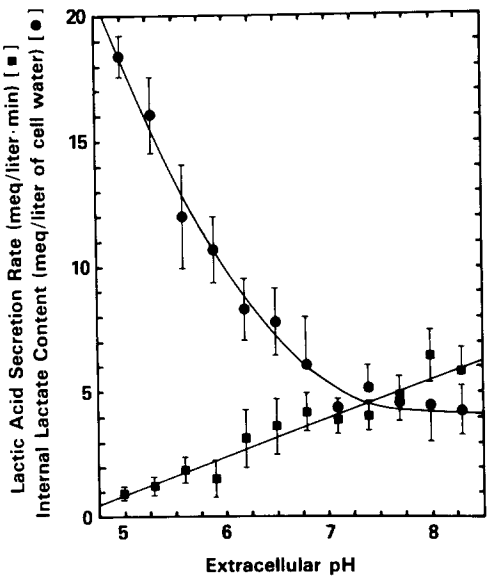

FIGURE 11. Extracellular pH dependence of lactic acid secretion by neutrophils and its effect on internal Lac levels. Experiments were performed in 2 mM Lac, 145 mM Cl^- medium in which the pH_o was varied between 5.0 and 8.3. For the efflux studies, the secretion of lactic acid into the medium was assessed at 5 and 10 min and the linear secretion rates were calculated by fitting the data points to straight lines. Parallel studies were also conducted in which the intracellular Lac contents were measured and the initial rates of Lac accumulation determined as in Fig. 9. Results are from four experiments. The data set for lactic acid secretion was empirically fit by a straight line for which the equation for the least-squares regression is: secretion rate (in meq/liter·min) = 1.5 (pH_o) − 6.8. For the intracellular Lac data points, the curve has been drawn by eye.

the empty carrier constitute the rate-limiting step? To elucidate this point, we measured the efflux of [^{14}C]Lac into 148 mM glucuronate medium lacking Lac (zero *trans*) and into 118 mM glucuronate medium containing 30 mM Lac (infinite *trans*). The experiments shown in Table III reveal that one-way efflux rates were remarkably

TABLE III

Effect of External Lac on the Rate of [^{14}C]Lac Efflux into Glucuronate Medium

$[Lac]_o$	Lac efflux
mM	*meq/liter · min*
0	5.4 ± 0.4
0 (+ Mersalyl)	0.10 ± 0.04
30	5.9 ± 0.5
30 (+ Mersalyl)	0.13 ± 0.02

Neutrophils were incubated with 6 μCi/ml of [^{14}C]Lac in 2 mM Lac, 145 mM Cl^- medium for 15 min. Thereafter, the cells were washed quickly at 4°C and resuspended in either Lac-free, 148 mM glucuronate, or 118 mM glucuronate medium containing 30 mM Lac, each in the presence or absence of 250 μM mersalyl. The radioactivity remaining in the cell pellet was measured at several times (5, 10, 15, 20, 25, and 30 s) and the rate coefficients were determined by fitting the data points to declining single exponential equations. In calculating the initial efflux rates, the internal Lac concentration at zero time was taken as 5.2 meq/liter of cell water (Fig. 9). Results are from three experiments.

similar under the two treatment conditions. These results indicate that the half-cycle reaction comprising the efflux translocation of Lac is most likely rate limiting.

The Lac Carrier Mediates H^+ + Lac^- Cotransport

The appearance of lactic acid in the medium bathing metabolically active neutrophils, together with the pH dependence of [^{14}C]Lac influx, is consistent with the notion that the carrier mediates H^+ + Lac^- cotransport or some phenomenologic equivalent. Positive proof that this is indeed the case is provided in Fig. 12. Here, we reasoned that if in fact proton movements were truly coupled to those of Lac, then an influx of H^+ in association with a net uptake of Lac should be detectable using pH-stat techniques. For these studies, the pH_o was maintained at 6.0 in order to

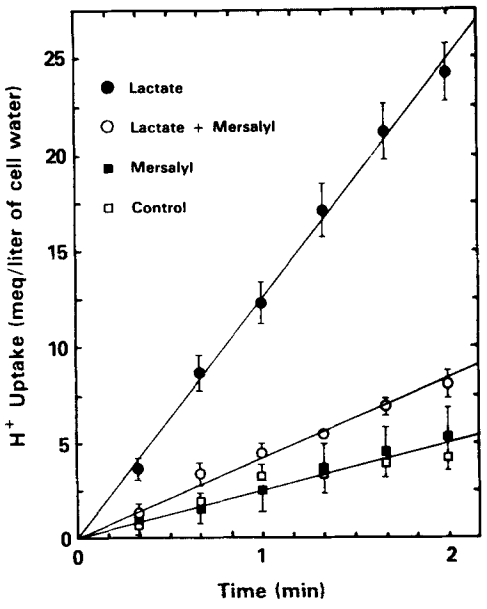

FIGURE 12. Direct demonstration that the Lac carrier mediates H^+ + Lac^- cotransport. Experiments were conducted in 145 mM Cl^- medium at pH_o 6.0 in order to enhance fluxes via the Lac transporter. In addition, the neutrophils were pretreated with 1 mM 2-DOG in glucose-free medium at pH_o 7.40 so as to block lactic acid production and reduce the internal Lac content to very low levels. A negligible $[Lac]_i$ together with the marked extracellular acidification provided a very favorable inward chemical driving force for net H^+ uptake along with an accompanying Lac anion. At zero time, 2 mM Lac or 0.25 mM mersalyl were added singly or in combination to the cell suspensions. The influx of H^+ into the cells was followed continuously by measuring the loss of acid equivalents from the bathing medium using a pH-stat device. The results of 5–10 experiments have been converted to milliequivalents per liter of cell water for the corresponding H^+ influx into the neutrophils. The data points were fit to straight lines with H^+ uptake rates (slopes) of 12.5 ± 0.4 (Lac), 4.2 ± 0.2 (Lac + mersalyl), and 2.5 ± 0.2 (combined mersalyl and control sets) meq/liter·min.

accelerate fluxes via the carrier and to provide a favorable driving force for inward movements of H^+ and Lac^-. As shown, there is little in the way of net proton fluxes in the control medium (145 mM Cl^-), either in the presence or absence of 0.25 mM mersalyl. That mersalyl by itself has no effect on the pH_o of poorly buffered medium implies that the drug is not transported inward via the carrier. However, the addition of 2 mM Lac to the bathing solution resulted in a dramatic rise in pH_o as H^+ equivalents entered the cells. That this proton influx occurs via the Lac transporter can be demonstrated by the fact that it can be >80% blocked by the simultaneous addition of mersalyl. It is of note that the H^+ influx rate of 12.5 ± 0.4 meq/liter·min

compares quite favorably with the rate of [^{14}C]Lac influx at pH_o 6.0 (Fig. 5 *A*), although the latter studies had been performed on cells with normal intracellular Lac. When the experiments were repeated, this time using 2-DOG-pretreated neutrophils at pH_o 6.0, a measured rate for [^{14}C]Lac influx of 11.8 ± 0.9 meq/liter·min was obtained, similar to the value for H^+ influx under identical conditions. This is as would be expected for an H^+ + Lac^- cotransport system of 1:1 stoichiometry, although coupling ratios of 2:1 or 3:2 cannot be ruled out.

The rate of H^+ influx into the cells at pH_o 6.0 was also a direct function of the concentration of external Lac (data not shown). The relationship could be adequately described by a Michaelis-Menten activation equation with a K_m (Lac) of 6.1 ± 1.9 mM, similar to the value of 5.4 ± 1.3 mM determined from the stimulation of [^{14}C]Lac influx (Fig. 6).

TABLE IV

Effects of External Glucose and Lac on the Steady Levels of Internal Lac and Rates of Lactic Acid Production and Secretion

External conditions	$[Lac]_i$	Lactic acid flux	
		Secretion	Production
	mM	*meq/liter · min*	
Control	5.5 ± 0.6	4.2 ± 0.4	3.0
Lac-free	4.7 ± 0.8	3.3 ± 0.3	3.3
Glucose-free	3.9 ± 0.5	2.4 ± 0.3	1.2
Lac- and glucose-free	1.8 ± 0.3	1.4 ± 0.2	1.4

Cells were incubated in the various experimental media at pH_o 7.40 for 1 h before taking measurements. The control medium contained the standard 2 mM Lac and 5.6 mM glucose. The intracellular Lac contents of the neutrophils (millimolar) and the lactic acid secretion rates (expressed in meq/liter · min) were determined as described in Materials and Methods. The production rate of lactic acid (also in meq/liter · min) was calculated as follows: taking the cells to be in steady state, and assuming no other relevant pathways for lactic acid, the production rate represents the difference between secretion and influx, the latter amounting to zero in Lac-free solutions and 1.2 meq/liter · min in 2 mM Lac medium. Results have been taken from five separate experiments.

Steady-State $(Lac)_i$

We also examined the factors that affect the steady level of intracellular Lac in human neutrophils. It seems reasonable to assume that rather than being precisely regulated at a fixed value, the $[Lac^-]_i$ represents a balance between the factors that tend to increase internal Lac (influx from the bathing solutions and metabolic production from glucose) and those that tend to decrease it (secretion into the medium). For these experiments, we lowered the amount of Lac influx by depleting the medium of Lac and reduced the biosynthesis of lactic acid via anaerobic glycolysis by prolonged (1 h) incubation of neutrophils in glucose-free medium. Table IV shows the new "steady-state" internal Lac contents as well as the secretion and production rates of lactic acid under these conditions. For control neutrophils bathed in a 2-mM Lac

medium containing 5.6 mM glucose, influx amounts to 1.2 ± 0.2 meq/liter·min, while lactic acid secretion is 4.2 ± 0.4 meq/liter·min. Note that the difference between one-way [^{14}C]Lac efflux and influx rates (6.3 − 1.5 = 4.8 meq/liter·min for net efflux; Figs. 2 and 3) is similar to the value of 4.2 meq/liter·min for secretion under standard conditions, thereby proving the cells to be in a true steady state. Therefore, for these steady-state cells, biosynthetic processes must contribute ~3.0 meq/liter·min since the sum of influx + production = secretion. When all Lac is left out of the medium, thereby reducing influx to zero, there is a significant, though rather modest decline in $[Lac]_i$ and lactic acid secretion, as would be expected since influx makes only a relatively minor contribution to total Lac kinetics. Thus, the steady level of internal Lac fell from 5.5 ± 0.6 to 4.7 ± 0.8 meq/liter of cell water and secretion fell from 4.2 to 3.3 ± 0.3 meq/liter·min, the calculated production rate remaining the same (3.0 vs. 3.3 meq/liter·min). By comparison, glucose depletion of the cells, which limits the metabolic production of lactic acid via glycolysis, has a much more pronounced effect. Cells kept in glucose-free medium in the presence of 2 mM Lac exhibited a $[Lac]_i$ of only 3.9 ± 0.5 meq/liter of cell water and a secretion rate of 2.4 ± 0.3 meq/liter·min. Assuming the cells to be in steady state, it follows that the production rate of lactic acid was only 1.2 meq/liter·min (2.4 minus the influx rate of 1.2) or ~40% of normal. As might be anticipated from the above results, the simultaneous depletion of Lac and glucose from the medium, thereby suppressing both processes that serve to elevate $[Lac]_i$, caused a marked reduction in all three parameters (Table IV).

DISCUSSION

Isolated human peripheral blood neutrophils bathed in a standard 145 mM Cl^- medium containing 5.6 mM glucose and 2 mM Lac, pH_o 7.40, secrete lactic acid at the rate of ~4 meq/liter·min. This flux is of the same order of magnitude as, though somewhat greater than, the total one-way fluxes of Na^+, K^+, and Cl^- (~1, ~1, and ~2 meq/liter·min, respectively) in resting, steady-state cells.

In this study, we presented evidence that lactic acid fluxes are mediated via a specialized H^+ + Lac^- cotransport system. The carrier is sensitive to mersalyl and UK-5099, both of which block fluxes in both directions by suppressing the transport velocity. However, the Lac carrier is resistant to several weak organic acid transport inhibitors such as ethacrynic acid, furosemide, SITS, and H_2DIDS. Other agents, namely, DISA, MK-473, DPC, and pentachlorophenol, which are themselves weak acids, inhibit Lac influx and efflux in short-term studies, but have no effect on lactic acid secretion after several minutes due to a dramatic and as yet unexplained loss of activity at the cytoplasmic surface. These compounds behave as competing substrates for the Lac transporter. Other substrates include aliphatic and aromatic monocarboxylates such as pyruvate, 2-chloroacetate, 2-chlorobenzoate, 3-hydroxybutyrate, and D-Lac, but not isethionate, glucuronate, or di- and tricarboxylates. In addition, the carrier has a rather low affinity for inorganic anions such as Cl^- and SO_4^{2-}.

Comparison with Other Anion Transport Systems in Neutrophils

Several lines of evidence indicate that Lac is not handled by the principal anion exchange mechanism (Cl^-/HCO_3^-) in these cells and that this countertransporter and

the H^+ + Lac^- cotransport system clearly represent separate and distinct entities: (*a*) lactic acid fluxes are approximately fourfold greater in magnitude than $^{36}Cl^-$ fluxes through the exchanger under comparable conditions (Simchowitz and De Weer, 1986); (*b*) external Lac (up to 40 mM) does not *trans*-stimulate $^{36}Cl^-$ efflux into an otherwise inert glucuronate medium (Simchowitz, L., unpublished observations); (*c*) external Lac (up to 40 mM) has no effect on the influx of $^{36}Cl^-$ from a 5 mM Cl^- medium, indicating that the major Cl^- carrier possesses little if any affinity for Lac (Simchowitz, L., unpublished observations); and (*d*) the affinity of the Lac transporter for Cl^- is low ($K_m \sim 100$ mM), while that of the Cl^-/HCO_3^- exchanger is high ($K_m \sim 5$ mM; Simchowitz, Ratzlaff, and De Weer, 1986). In addition, it is equally evident from the fact that Lac fluxes are insensitive to ethacrynate, SITS, and furosemide that Lac cannot be handled via the neutrophil's major SO_4^{2-} carrier. As we have recently reported (Simchowitz and Davis, 1989), this transport system behaves as an H^+ + SO_4^{2-} cotransport, the ion pair being translocated across the cell membrane in exchange for Cl^- as in erythrocytes (Jennings, 1976; Milanick and Gunn, 1982).

With respect to inhibitors, H^+ + Lac^- cotransporters seem to be uniformly sensitive to aromatic monocarboxylates, particularly cyanocinnamate derivatives like CHC and other related analogues (Halestrap and Denton, 1975; Deuticke, 1989). However, it is important to stress that these compounds also block Cl^-/HCO_3^- exchange in erythrocytes and neutrophils (Deuticke, 1982; Simchowitz, 1988). It is of note, and perhaps not a coincidence, that all of the agents reported herein that inhibit lactic acid transport also block $^{36}Cl^-$ fluxes through the Cl^-/HCO_3^- exchanger (Simchowitz, L., unpublished data). In red cells, sulfhydryl group-modifying mercurials and thiol compounds have little effect on the inorganic anion exchanger and can be used to discriminate between the two pathways (Deuticke, 1982, 1989). So too, in neutrophils, we have observed that *p*-hydroxymercuribenzoate, 5,5′-dithiobis(2-nitrobenzoate), and 2,2′-dithiobispyridine (Aldrithiol) block [^{14}C]Lac fluxes while having negligible effects on $^{36}Cl^-$ fluxes via the anion exchanger (Simchowitz, 1988, and Simchowitz, L., unpublished observations). Since most of the effective drugs behave as competitive inhibitors of Lac and Cl^- at the external translocation sites of their respective carriers, it would appear that the anion recognition units are similar to a degree (or at least that portion which seems to bind the drugs). Perhaps we are dealing with two related proteins that, during evolution, ultimately derive from the same ancestral carrier gene and therefore constitute two members of an extended family of transport proteins. Of course, this issue can only be satisfactorily resolved when the protein biochemistry and molecular biology of these anion transport systems have been successfully elucidated.

Kinetic Considerations

In the short-term experiments of Figs. 2 and 3, mersalyl, DISA, UK-5099, and pentachlorophenol are each equally efficacious in blocking the cross-traffic of Lac in both directions; i.e., one-way [^{14}C]Lac influx and efflux are likewise altered. It is of note that all of these drugs except mersalyl readily cross the plasma membrane in the free acid form via simple nonionic diffusion; none is transported inward via the Lac carrier. This movement can be detected from the associated proton influx measured using pH-stat techniques (Simchowitz, Textor, and Vogt, 1991). Thus, their entry

into the cytosol should make these agents available to the intracellular surface of the transporter. On the basis of this evidence alone, it is difficult to predict whether or not the drugs are preferentially acting from one side or the other or both. In contrast, mersalyl is impermeant and does not gain access to the cytosol, thereby indicating that this agent in all likelihood exerts its effects exclusively from the outside.

A further complexity in the analysis has been added by the surprising finding that after 1 min there is a striking loss of inhibition of [^{14}C]Lac efflux with DISA such that lactic acid secretion rates are normal by 5, 10, and 20 min of incubation. This occurs despite the fact that the marked suppression of [^{14}C]Lac influx persists throughout this time period. Whatever the features of this reversal mechanism, they seem to be shared by pentachlorophenol and DPC, and to a lesser extent by UK-5099. Carrier asymmetry, wherein the internal translocation site displays a lower affinity for the inhibitor, is theoretically possible, but other factors must also be invoked. Allosteric regulation of the transport protein could be involved, especially since a prominent cytoplasmic acidification is known to occur during the first few minutes of exposure of neutrophils to these drugs (Simchowitz et al., 1991).

In contrast, mersalyl is not affected in this manner, its inhibition of Lac carrier function being sustained. Perhaps this may be related to unique structural elements of the compound: the ability of this organomercurial to interact not only with the substrate binding site (a property shared equally with DISA, UK-5099, and pentachlorophenol), but also with a critical sulfhydryl group. Given the incompleteness of our knowledge at this time, it would seem that several other models could explain the experimental observations.

pH Effects

Over the years, a general consensus has emerged that monocarboxylate carriers function as H^+ + anion X^- cotransport systems. Indeed, several comprehensive discussions and reviews have appeared (Spencer and Lehninger, 1976; Regen and Tarpley, 1978, 1980; Deuticke, 1982) wherein mobile carrier models have been constructed to satisfactorily resolve the wealth of experimental observations. The effects of pH on Lac kinetics reported herein for neutrophils are entirely consistent with data derived from other cell types and with current concepts regarding the reaction scheme. While allosteric effects of H^+ on the carrier protein cannot be completely ruled out, all of the observed effects of pH can be ascribed to the role of H^+ as a direct participant in the transport reaction. Thus, (*a*) the influx of Lac results in extracellular alkalinization, (*b*) extracellular acidification reduces secretion, whereas alkalinization facilitates the efflux of lactic acid, and (*c*) extracellular acidification promotes Lac entry while intracellular acidification reduces Lac influx. That the *cis* and *trans* effects of H^+ are opposite, a prediction of the models, is a point in keeping with the notion that the pH interactions truly take place at the substrate translocation site rather than at some modifier site on the protein. The latter would be expected to alter reactions equally in both directions.

Of note is the characteristic feature that external acidification causes a shift in the half-saturation constant for monocarboxylate influx in favor of higher affinity, the K_m generally falling by a factor of ~2–3 for a 1 U reduction in pH_o (Dubinsky and

Racker, 1978; Regen and Tarpley, 1978; Deuticke, 1982). The finding that K_m (Lac) in human neutrophils is 5.4 mM at pH_o 6.30 as compared with 13.0 mM at pH_o 7.40 is in conformity with these results. As analyzed by Regen and Tarpley (1980), this stems from the fact that under conditions of low substrate concentration as in our studies, extracellular acidification sequesters the carrier in an outward-facing orientation. Therefore, virtually all the carrier is exposed to the substrate and is in a form that can readily translocate inward after anion binding, the net result being a lowering of entry K_m. Other explanations for this phenomenon are possible. The complex effects of pH on K_m for Lac transport are no doubt model dependent and thus a true conceptual understanding requires a more thorough kinetic analysis. These considerations serve to emphasize, however, that the general properties of the Lac carrier of human neutrophils are remarkably similar to those in other tissues.

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