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KINETIC ANALYSIS OF L-LACTATE TRANSPORT IN HUMAN ERYTHROCYTES VIA THE MONOCARBOXYLATE-SPECIFIC CARRIER SYSTEM

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Three parallel pathways of L-lactate transport across the membrane of human red blood cells can be discriminated: (a) by nonionic diffusion; (b) via the band 3 anion exchange protein; and (c) via a specific monocarboxylate carrier system. Influx of lactate via the latter system leads to alkalinization of the medium, suggesting lactate-proton symport. Kinetic analysis of initial lactate influx via the monocarboxylate carrier indicates a symport system with ordered binding of the two ligands, in the sense that a proton binds first to the translocator, followed by lactate binding to the protonated carrier. The influence of varying trans-pH under conditions of net (zero-trans) flux with constant cis-pH indicates that the monocarboxylate translocator should be considered as a mobile carrier, with the ligand-binding sites exposed alternately to the outside and the inside of the membrane.

Introduction

Monocarboxylate transport across the red cell membrane has been the subject of several recent studies [1–6]. It has been shown that L-lactate can cross the human red cell membrane via three different pathways. Nonionic diffusion accounts for about 5% of total lactate movement under physiological conditions. As shown by Deuticke et al. [4] this strongly pH-dependent passive diffusion is accelerated by hexanol, indicating diffusion via the lipid bilayer. A second minor transport pathway, also accounting for about 5% of lactate transport, proceeds via the band 3 anion exchange protein [4,5]. This transport can be completely blocked by DIDS, a specific inhibitor of band 3 activity [7,8]. The remaining, roughly 90% of lactate transport occurs via a specific monocarboxylate carrier, which is highly sensitive to SH-reagents like PCMBS [2]. This major pathway of lactate transport in human erythrocytes exhibits the characteristics of a lactate-proton symport system, or its phenomenological equivalent, a lactate-OH^- antiport system [1,5]. The alkalinization of the medium during lactate influx and the observed stimulation of influx by decreasing medium pH [1] can also be reconciled, however, with a facilitated diffusion system for undissociated lactic acid.

Substrate-proton symport systems are well-known in bacteria [9,10], algae [11,12] and yeasts [13,14]. In these cases the electrochemical H^+-gradient across the cell membrane provides the energy for uphill transport of the substrate, according to the chemiosmotic coupling hypothesis of Mitchell [15]. In recent studies it has been shown that kinetic analysis of initial transport can be used to discriminate between the possible reaction types of the symport carrier, viz., random binding, or obligatory ordered binding with either substrate or proton binding first [16,17]. More-

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; PCMBS, *p*-chloromercuriphenylsulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

over, such analysis may indicate whether the transporter should be considered as a mobile carrier, with the ligand-binding sites exposed alternately to the two membrane sides, or as a non-mobile carrier [18], with fixed binding sites at each side of the membrane.

In the present communication, a similar kinetic analysis of initial transport of lactate via the monocarboxylate carrier of human erythrocytes is presented, as an attempt to describe the activity of this carrier on the molecular level.

Methods

A 20% suspension of red blood cells in isotonic NaCl, buffered with 20 mM tricine at pH 8.0, was incubated for 30 min at 37°C and subsequently washed twice in isotonic NaCl/10 mM Hepes (pH 7.5). With this pretreatment, virtually all intracellular lactate was removed.

For initial influx measurements, a 30% cell suspension was further preincubated during 1 min with 14 μM DIDS to block band 3 activity. Subsequently, the pH was adjusted to the desired value and influx was initiated 1 min later by addition of a ^{14}C-labeled L-lactate solution. At 0.5, 1.25, 2.00 and 2.75 min, 180-μl samples were withdrawn and layered on 0.2 ml di-*n*-butylphthalate in a 0.5 ml microcentrifuge tube. The cells were spun down through the di-*n*-butylphthalate layer at 6000 rpm in an Eppendorf 3200 centrifuge. The tip of the centrifuge tube, containing the red blood cells, was cut off and treated with 1 ml H_2O_2 in a scintillation vial. Radioactivity was measured by liquid scintillation counting.

During these experiments, intracellular and medium pH are constant. As shown previously by Deuticke et al. (Ref. 4, and Ref. 40 therein) no readjustment of pH can be detected in short-term experiments with DIDS-treated cells. In control experiments this could be confirmed. Initial influx velocity via the monocarboxylate carrier was calculated from the slope of the uptake curves, after correction for nonionic passive diffusion. This transport component was measured after treatment of the cells with both DIDS (to block band 3 activity) and PCMBS (to block the monocarboxylate carrier), as described by Deuticke et al. [4].

To measure final steady-state accumulation levels, incubations had to be continued for about 30 min. To ascertain stable medium pH during this period, the pH was adjusted in isotonic sucrose/KCl mixtures before addition of DIDS, as described by Wieth et al. [19].

For efflux measurements, cells were preloaded with [^{14}C]lactate and resuspended in 100 vol. isotonic medium of the desired pH. Efflux was monitored by measurement of radioactivity in the supernatant.

Proton influx was monitored by titration with 0.001 M HCl, utilizing a Mettler DK automatic titration equipment under pH-stat conditions.

L-[^{14}C]Lactate was obtained from Amersham International. DIDS was purchased from Pierce, PCMBS from Sigma.

Theoretical

The observed electroneutrality of lactate influx via the monocarboxylate carrier can theoretically be explained in three ways: (1) lactate-proton symport; (2) facilitated diffusion of undissociated lactic acid; or (3) lactate-OH^- antiport. These models are shown in Fig. 1. With the assumptions: (1) the translocator is in equilibrium with ligands in solution; (2) the H^+ (OH^-)/lactate stoichiometry is 1; and (3) the total amount of ligand binding sites is constant, the models represent mobile carrier systems, with the binding sites exposed alternately to the two sides of the membrane. As shown previously [17] the kinetic parameters for initial influx ($S_i = 0$) for substrate-proton symport can be derived for the cases of random H^+ and substrate binding to the translocator and of ordered binding, with either H^+ or substrate binding first. These parameters are summarized in Table I. Along similar lines, the kinetic parameters for facilitated diffusion of lactic acid and lactate-OH^- antiport can be derived (Table I).

The models shown in Fig. 1 would represent non-mobile carrier systems [18], under the additional assumption that the total number of ligand-binding sites at each membrane-solution interface is constant. On the molecular level, such a non-mobile carrier system can be visualized as a translocator, spanning the membrane, with fixed substrate binding sites at each side of the membrane. Transport of the bound substrate follows, for ex-

Lactate acid (L_t): $HL \overset{K_L}{\rightleftharpoons} H^+ + L^-$

$$[L_t] = \frac{[HL](K_L + [H^+])}{[H^+]} = \frac{[L^-](K_L + [H^+])}{K_L}$$

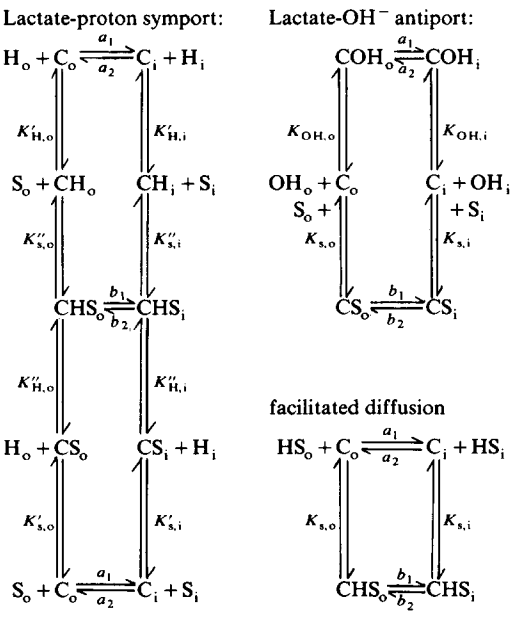

Fig. 1. Kinetic models of monocarboxylate transport in red blood cells. $C_o \ldots CHS_o$, $C_i \ldots CHS_i$, ligand-binding sites facing, respectively, the outside and inside of the cell; H, proton, S, monocarboxylate; K, dissociation constant; $a_1 \ldots b_2$, translocation velocity constants.

ample, by shifting of the substrate molecule from one to the other binding site, from which it is subsequently released at the other side of the membrane (see Ref. 18). The kinetic parameters, as derived previously [16], are summarized in Table II.

As all experiments were performed in the pH range 5.5–8.7, the term $(K_L + H_o)/K_L$ in the equations in Tables I and II is very close to 1 ($K_L = 10^{-3.86}$) and can be neglected.

Results

In preliminary experiments the three pathways for lactate transport in human erythrocytes were confirmed. In all further experiments, influx via the monocarboxylate carrier was calculated from the difference of fluxes into DIDS-treated cells and (DIDS + PCMBS)-treated cells.

The H^+ influx (or OH^- efflux) during lactate uptake is depicted in Fig. 2. Parallel measurements of lactate influx revealed a 1 : 1 H^+-lactate stoichiometry, in accordance with previous results of Dubinsky and Racker [1].

A final accumulation ratio of lactate should be expected according to the Donnan-equilibrium:

$$\frac{[S_i]}{[S_o]} = \frac{[H_o]}{[H_i]}$$

TABLE I

THE KINETIC PARAMETERS FOR MOBILE CARRIER MODELS DURING INITIAL INFLUX

	K_{app}	V_{app}
Symport, random	$\frac{K'_{s,o}K''_{H,o}\{a_2K'_{H,i}(K'_{H,o}+H_o)+a_1K'_{H,o}(K'_{H,i}+H_i)\}}{K'_{H,o}\{a_2K'_{H,i}(K''_{H,o}+H_o)+b_1H_o(K'_{H,i}+H_i)\}} \cdot \frac{K_L+H_o}{K_L}$	$\frac{a_2b_1C_TK'_{H,i}H_o}{a_2K'_{H,i}(K''_{H,o}+H_o)+b_1H_o(K'_{H,i}+H_i)}$
Symport, ordered, H^+ binds first	$\frac{K''_{s,o}\{a_2K'_{H,i}(K'_{H,o}+H_o)+a_1K'_{H,o}(K'_{H,i}+H_i)\}}{H_o\{a_2K'_{H,i}+b_1(K'_{H,i}+H_i)\}} \cdot \frac{K_L+H_o}{K_L}$	$\frac{a_2b_1C_TK'_{H,i}}{a_2K'_{H,i}+b_1(K'_{H,i}+H_i)}$
Symport, ordered, S binds first	$\frac{K'_{s,o}K''_{H,o}(a_1+a_2)}{a_2(K''_{H,o}+H_o)+b_1H_o} \cdot \frac{K_L+H_o}{K_L}$	$\frac{a_2b_1C_TH_o}{a_2(K''_{H,o}+H_o)+b_1H_o}$
Antiport	$\frac{K_{s,o}\{a_2OH_i(K_{OH,o}+OH_o)+a_1OH_o(K_{OH,i}+OH_i)\}}{K_{OH,o}\{a_2OH_i+b_1(K_{OH,i}+OH_i)\}} \cdot \frac{K_L+H_o}{K_L}$	$\frac{a_2b_1C_TOH_i}{a_2OH_i+b_1(K_{OH,i}+OH_i)}$
Facilitated diffusion	$\frac{K_{s,o}(a_1+a_2)}{a_2+b_1} \cdot \frac{K_L+H_o}{H_o}$	$\frac{a_2b_1C_T}{a_2+b_1}$

TABLE II

THE KINETIC PARAMETERS FOR NON-MOBILE CARRIER MODELS DURING INITIAL INFLUX

	K_{app}	V_{app}
Symport, random	$\frac{K'_{s,o}K''_{H,o}(K'_{H,o}+H_o)}{K'_{H,o}(K''_{H,o}+H_o)} \cdot \frac{K_L+H_o}{K_L}$	$\frac{b_1C_TH_o}{K''_{H,o}+H_o}$
Symport, ordered H^+ binds first	$\frac{K''_{s,o}(K'_{H,o}+H_o)}{H_o} \cdot \frac{K_L+H_o}{K_L}$	b_1C_T
Symport, ordered, S binds first	$\frac{K'_{s,o}K''_{H,o}}{K''_{H,o}+H_o} \cdot \frac{K_L+H_o}{K_L}$	$\frac{b_1C_TH_o}{K''_{H,O}+H_o}$
Antiport	$\frac{K_{s,o}(K_{OH,o}+OH_o)}{K_{OH,o}} \cdot \frac{K_L+H_o}{K_L}$	b_1C_T
Faciliated diffusion	$K_{s,o} \cdot \frac{K_L+H_o}{H_o}$	b_1C_T

As shown in Fig. 3, this is born out experimentally. When the Donnan-equilibrium is disturbed, for example, by addition of valinomycin, a prompt readjustment of the $[S_i]/[S_o]$ ratio occurs (Fig. 4).

Further experiments were designated to determine the mechanism of lactate transport by the monocarboxylate transporter. Therefore, the influence of medium pH on the kinetic parameters of initial influx was measured. As shown in Fig. 5, V_{app} is pH-independent. Despite the relatively large experimental error, it is evident that there is no tendency for a V_{app}-change over the pH range 5.5–8.7. The K_{app}, however, is strongly pH-dependent, as shown in Fig. 6. These experimental results rule out facilitated diffusion of undissociated lactic acid. The pH-dependency of K_{app} for this mode of transport is determined by the value of $(K_L + H_o)/H_o$ (Tables I and II). The expected pH-dependency for this transport system is shown in Fig. 6 by the dashed line, which is irreconcilable with the experimental results. The pH-independent V_{app} combined with a strongly pH-dependent K_{app}

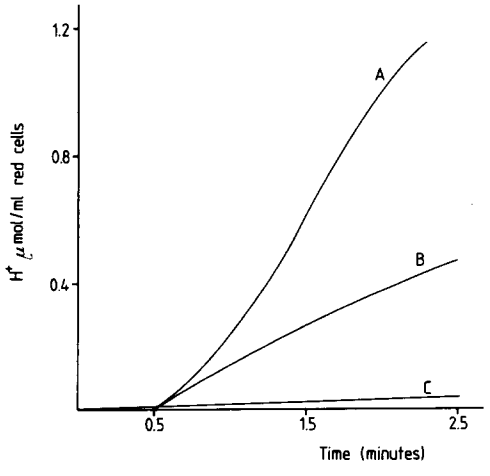

Fig. 2. Proton influx during lactate uptake at pH 6.5. Erythrocyte concentration, 10%; lactate concentration, 6.5 mM. A, DIDS-treated cells in the presence of lactate; B, (DIDS+PCMBS)-treated cells in the presence of lactate; C, control, DIDS-treated cells without substrate. DIDS was added at $t = 0$, lactate at $t = 0.5$ min.

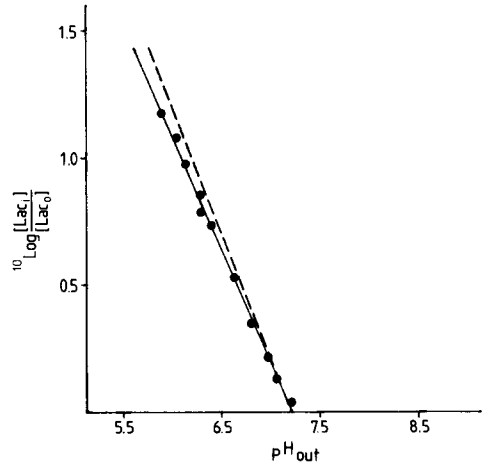

Fig. 3. The relationship between the lactate accumulation ratio after 40 min incubation at 25°C with 0.05 mM lactate, and the external proton concentration. Dashed line: theoretical curve, assuming Donnan-equilibrium, with pH_i 7.35.

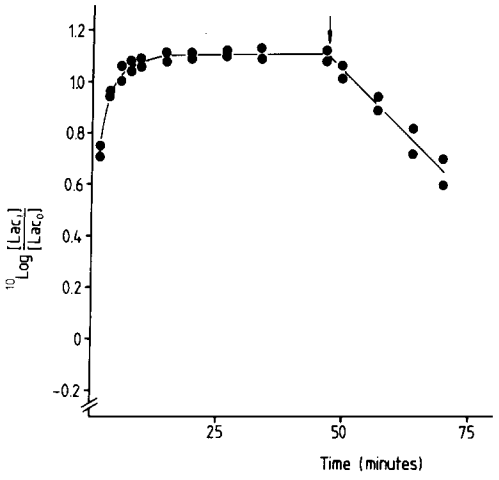

Fig. 4. Lactate accumulation at pH 6.1. After 47.5 min, valinomycin was added (final concentration, 5 μM). Lactate concentration, 0.05 mM. Temperature, 25°C.

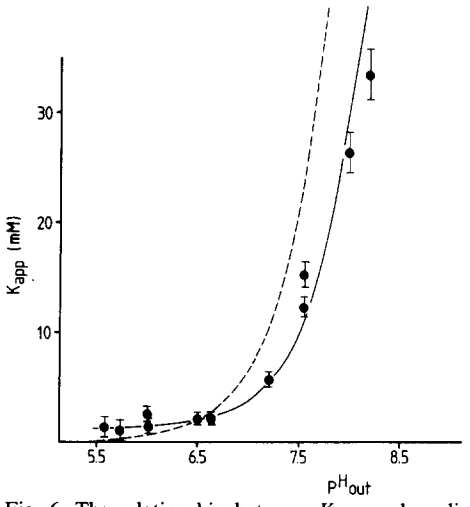

Fig. 6. The relationship between K_{app} and medium pH. The solid line represents the best-fitting curve, with $K'_H = 10^{-6.7}$. Dashed curve: theoretical curve of facilitated diffusion of undissociated lactic acid, drawn through the point pH 6.5.

indicates either lactate-proton symport, with ordered ligand binding, proton binding first, or lactate-OH^- antiport.

From Tables I and II it appears that in (zero-trans) flux measurements the kinetic parameters of mobile carrier symport systems are sensitive to variations of the trans-pH, whereas the parameters for non-mobile carrier systems are not. The influence of trans-pH was studied in (zero-trans) efflux experiments, as it is much easier to vary the medium pH, instead of the intracellular pH. The experiments were performed at very low lactate concentrations. Under these conditions $v/[s]$ is a direct measure of V_{app}/K_{app}. As shown in Fig. 7, this parameter is clearly pH-dependent.

Discussion

The presence of a specific monocarboxylate carrier in erythrocyte membranes has been demon-

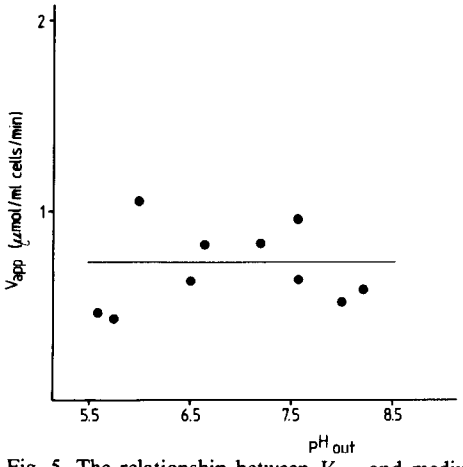

Fig. 5. The relationship between V_{app} and medium pH. V_{app} was deduced from initial influx measurements, as described in the Methods section.

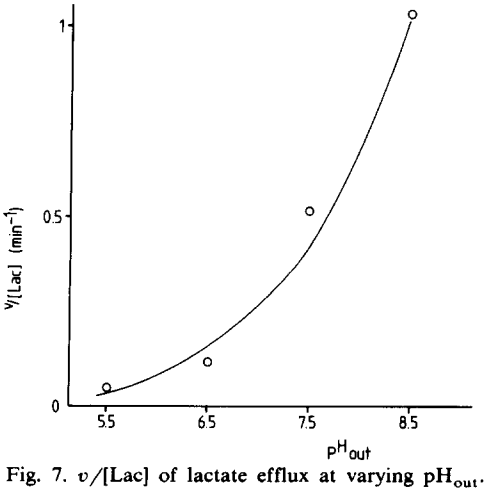

Fig. 7. $v/[Lac]$ of lactate efflux at varying pH_{out}. The cells were preloaded at an overall lactate concentration of $5.2 \cdot 10^{-5}$ M.

strated irrefutably by previous investigations [1–5,20,21]. The strictly coupled H^+ influx (or OH^- efflux) during lactate uptake via this specific carrier can, theoretically, be accounted for by any by the models, presented in the theoretical section.

At the molecular level each of these models may represent a translocator, operating either as a mobile carrier or as a non-mobile carrier, as defined, among other, by Borst-Pauwels [18]. As shown in Table II the kinetic parameters for all presented non-mobile carrier models are insensitive to the trans-pH value, when measuring zero-trans fluxes. The results, presented in Fig. 7, however, clearly show a trans-pH dependency of V_{app}/K_{app}, indicating that the monocarboxylate carrier operates in a mobile carrier fashion. In this respect the monocarboxylate carrier differs from the proton-galactoside symporter, the proton-sorbose symporter and the Rb^+ translocator in yeast cells. These three transport processes proceed via a non-mobile carrier mechanism, as shown previously [16,17,22].

The experimental results exclude the possibility of facilitated diffusion of undissociated lactic acid. First of all, this transport mechanism would be insensitive to variations of trans-pH (Tables I and II). Indeed, aspecific effects of trans-pH on facilitated diffusion can not be excluded, but it seems highly improbable that aspecific effects would generate the curve shown in Fig. 7, which would, in that case, indicate a pH-optimum of the transport system far beyond pH 9.0. Further, curve fitting of K_{app} with the calculated values of $(K_L + H_o)/H_o$ shows that also in this respect facilitated diffusion is irreconcilable with the experimental data (see Fig. 6, dashed curve).

On the proton-lactate symport systems, the random binding and ordered binding with lactate as leading substrate are contradicted by the experimental results. In both cases, the V_{app} would have been dependent on the external pH (Table I). In a previous paper it has been argued that a pH-independent V_{app} is still compatible with random binding, assuming simplifying conditions [5]. Supposing a symmetric system ($a_1 = a_2 = a$, $K'_{H,o} = K'_{H,i} = K'_H$, etc.), V_{app} for a random binding system becomes indeed more or less pH_o-independent, when $K''_H/[H_o] \ll 1 + b/a(1 + [H_i]/K'_H)$ [5]. This latter assumption is very unlikely, however, based on the following considerations. The best-fitting curve, as drawn in Fig. 6, corresponds to a K'_H value of $10^{-6.7}$. The trans-acceleration of lactate transport by lactate indicates $b > a$. From experimental results, presented by Deuticke [3], an upper value of $b/a = 6$ can be deduced. Thus, with $[H_i] = 10^{-7.35}$, the upper value of $1 + b/a(1 + [H_i]/K'_H)$ can be estimated as 8.3. Assuming that a 30% increase of V_{app} over the pH range 5.5–8.7 might have escaped attention, due to the experimental error, K''_H should have a value of $10^{-8.1}$ or less, meaning a K''_H/K'_H (and thus also a K''_s/K'_s) ratio of 0.04 or less. This would imply a random ligand binding to the carrier with, at least a 25-fold change of the dissociation constant for the second ligand, subsequent to binding of the first ligand. Such a mechanism is very difficult to visualize at the molecular level. Therefore it seems justified to exclude a proton-lactate symport system with random binding.

It is impossible to discriminate, on a kinetic basis, between the two remaining possibilities, viz. proton-lactate symport with ordered binding, proton binding first, and lactate-OH^- antiport. The best-fitting curve (Fig. 6) corresponds to a K'_H value of $10^{-6.7}$ for the symport system, or a K_{OH} of $10^{-7.3}$ for the antiport system. In both cases, it can be calculated that K_s is about 6.9 mM. However, on molecular grounds, a lactate-OH^- antiport system seems very unlikely. As shown by several authors, the monocarboxylate carrier exhibits affinity for many monocarboxylates, but not for other anions. Considering the carrier as a lactate-OH^- antiport system, the only exception would be the OH^- ion, for which the carrier would have a $1.4 \cdot 10^5$-times higher affinity than for lactate. As this seems to be most improbable, the monocarboxylate carrier is most likely a lactate-proton symport system, with ordered binding, proton binding first.

It is noteworthy that other substrate-proton symport systems studied thus far by kinetic analysis, namely sorbose and galactoside transport in yeast [10,17], galactoside transport in *Escherichia coli* [23] and sulfate transport via band 3 in red blood cells [24] exhibit random binding of ligands to the carrier. In this context, kinetic analysis of other proton-substrate symport systems would be interesting.

The conclusion of ordered binding, proton binding first in the case of the monocarboxylate carrier has an intriguing consequence. It seems conceivable that H^+-binding to an uncharged group (e.g., a histidine residue) actually creates the binding site for the negatively charged lactate ion. Such a possibility would not have to be considered in the case of random binding.

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