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The Mechanism of Lactate Transport in Human Erythrocytes

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Summary. Lactate accumulates in human erythrocytes stored at 4 °C in the presence of glucose. Efflux of lactate exhibits an activation energy of 22 kcal/mole and is markedly stimulated with increasing medium pH. Lactate influx into erythrocytes that were depleted of intracellular lactate by incubation at 37° at pH 8.0 was stimulated by decreasing medium pH. Under appropriate conditions the pH-dependent lactate flux was insensitive to 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene or 4,4′-diisothiocyano-2,2′-disulfonic stilbene, inhibitors of the inorganic anion channel, while, e.g., inorganic phosphate transport was fully sensitive. These experiments as well as measurements of H^+ movements associated with lactate fluxes demonstrate that lactate transport takes place via a specific monocarboxylate transporter (distinct from the inorganic ion channel) by a H^+-lactate symport mechanism.

The transport of several small monocarboxylic acids across the plasma membranes of a variety of cells takes place on specific transport systems and not by simple diffusion of the free acid [4, 5, 11]. The mechanism of lactic acid transport in ascites tumor cells and *S. faecalis* is consistent with a lactate-proton symport or lactate/hydroxyl exchange [5, 11]. The transport system in ascites cells as well as that in human red cells is able to transport several small organic acids, e.g., pyruvate, lactate and glycolate [3, 4, 11]. In human erythrocytes two kinetically distinguishable pathways have been proposed, a monocarboxylate/Cl^- and a pyruvate/lactate exchange [4]. The former pathway was inhibited by 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene (SITS)[1]; the latter was not [4]. In recent studies Rice and Steck [8] showed that the initial rate of pyruvate

1 *Abbreviations used:*
DIDS – 4,4′-diisothiocyano-2,2′-disulfonic stilbene
SITS – 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene
HEPES – N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
MES – 2[N-morpholino]ethanesulfonic acid
TBT – tri-N-butyltin chloride
ACS – aqueous counting scintillant, Amersham Corp.

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flux into resealed ghosts from human erythrocytes was depressed in the presence of several inhibitors of the anion channel, including SITS, 1-fluoro-2,4 dinitrobenzene and phloretin. They conclude that their experiments "support the premise that pyruvate shares a common transport system with Cl^- and other anions."

On the other hand, sensitivity of lactate and glycolate equilibrium exchange in human erythrocytes to SH reagents of the mercurial type has provided evidence for a pathway of monocarboxylate transport which is distinct from the inorganic anion channel [3].

These apparently conflicting findings were resolved in the present study. Net lactate flux was measured in human erythrocytes with an imposed pH gradient. Under the conditions of these experiments, lactate transport occurred primarily via a monocarboxylate-specific transport system. Lactate translocation was associated with H^+ movements and was strongly pH dependent. Therefore, when a flux of Cl^- took place in the same direction, lactate translocation was inhibited; when Cl^- moved in the opposite direction, lactate transport was stimulated. Our results explain the observations on the effect of inhibitors of the inorganic anion transporter, such as SITS, and demonstrate that translocation of lactate in human erythrocytes takes place by a H^+-symport or OH^--antiport mechanism via a specific monocarboxylate transporter.

Methods and Materials

Whole blood was obtained from human donors by venipuncture. Each 10 ml of blood was treated with 1.5 ml of ACD solution (2.2% sodium citrate, 0.8% citric acid, and 2.45% dextrose) and stored at 0–4 °C.

Lactate Efflux Measurements

After 3–5 days of storage of whole blood the erythrocytes accumulated internal lactate. Cells were then washed in 5 volumes of 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$ and 10 mM HEPES (Na^+ salt pH 7.4) and were centrifuged for 5 min at 3,000 × *g* at 4 °C. The supernatant and the light buffy coat were aspirated. After three additional washings in the same volume the cells were suspended in an equal volume of buffer. Efflux was initiated by diluting an aliquot of these erythrocytes into 25 volumes of 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$ and 10 mM MES (pH 5–7) or 10 mM Tricine (pH 7.5–8.5). After various times of incubation indicated in the figure legends the reaction was terminated by dilution with an equal volume of ice cold 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM MES, pH 5.0. The cells were sedimented for 5 min at 3,000 × *g* at 4 °C. The pellet was lysed in 1 ml water and deproteinized with 0.4 ml of 2 M $HC1O_4$ and centrifuged for 5 min at 1,800 × *g*. The supernatant (0.8 ml) was neutralized with 0.2 ml of 2 M KOH,

0.4 M KCl and 0.4 M imidazole. The white precipitate ($KClO_4$) was sedimented at $1,800 \times g$ for 5 min and the supernatant was analyzed for lactic acid enzymatically [6].

Influx of Radioactive Lactate or Phosphate

Whole blood was depleted of accumulated lactate by incubation for 30 min at 37 °C in 20 to 40 volumes of chloride buffer (150 mM NaCl, 10 mM Na-Tricine, pH 8.0). The cells were sedimented at $3,000 \times g$ for 5 min. After three additional washes in either chloride buffer, citrate buffer (74 mM Na Citrate, 10 mM Na-Tricine, pH 8.0) or sucrose buffer (250 mM sucrose, 10 mM Na-Tricine, pH 8.0), the cell pellet was suspended with an equal volume of the corresponding buffer. Influx was initiated by addition of 25 µl of erythrocytes to 75 µl of buffer containing the indicated concentrations of ^{14}C-lactic acid (100–200 cpm/nmol) or $^{32}P_i$ (400–800 cpm/nmol) as described in the legends. After incubation for the indicated time, the reaction was terminated by addition of 5.0 ml of ice cold 0.15 M NaCl, 10 mM Na-MES, pH 6.5, and the mixture was centrifuged for 5 min at $3,000 \times g$ at 4 °C. After one wash in the same buffer, the pellet was lysed in 1 ml of water and deproteinized with 0.1 ml of 50% trichloroacetic acid. The mixture was centrifuged at $3,000 \times g$ for 5 min. The entire supernatant was added to scintillation vials and counted in 10 ml of ACS.

Measurements of pH

Changes in pH were monitored with a Beckman pH electrode and recorded on an Esterline Angus strip chart recorder.

Materials

L-(U-^{14}C)-lactate was obtained from New England Nuclear, Boston, Mass.; $^{32}P_i$ from ICN, Irvine, Calif.; L-lactic acid Grade L-1, and lactic acid dehydrogenase Type I were obtained from Sigma Chemical Co., St. Louis, Mo.; 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene (SITS) and 4,4′-diisothiocyano-2,2′-disulfonic stilbene (DIDS) from Pierce Chemical Co., Rockford, Ill.; tri-N-butyltin chloride from Aldrich Chemical Co., Metuchen, N.J.; aqueous counting scintillant (ACS) from Amersham Corp., Arlington Heights, Ill.

Results and Discussion

Lactate Efflux

Human erythrocytes stored at 4 °C with ACD solution accumulated lactate over several days (Table 1). Lactate accumulation was allowed to procede until the internal concentration was at least 3 to 5 mM. The lactate was retained when the washing was performed at 4 °C; however, brief incubation at 37 °C at a slightly alkaline pH caused a rapid loss of accumulated lactate. Measurements of efflux at different temperatures yielded an activation energy of about 22 kcal/mol from 15–35 °C

Table 1. Lactate accumulation during storage of erythrocytes

Time at 4 °C (days)	nmol lactate/mg protein
0	0.5
1	4.8
2	6.2
3	13.4
6	25.3

Erythrocytes were stored in ACD solution and washed prior to assay as described under *Materials and Methods*.

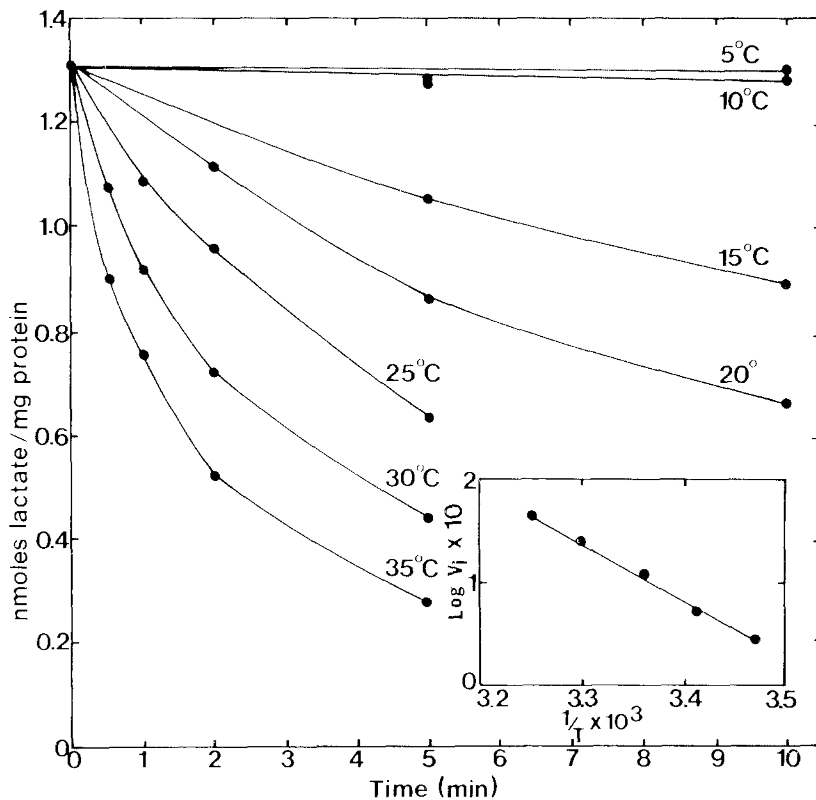

Fig. 1. Effect of temperature on lactate efflux. Erythrocytes were assayed for lactate efflux at the indicated temperatures in chloride buffer at pH 8 as described under *Materials and Methods*. Inset: Arrhenius plot of initial rates of efflux at different temperatures. Initial rate, V_i equals pmol lactate/mg protein · min

(Fig. 1). A somewhat higher value was obtained from 5–10 °C but could not be determined accurately due to the very low rates of efflux. The value is in the range of those reported for pyruvate entry into human erythrocytes [4], resealed ghosts [8] and ascites tumor cells [11].

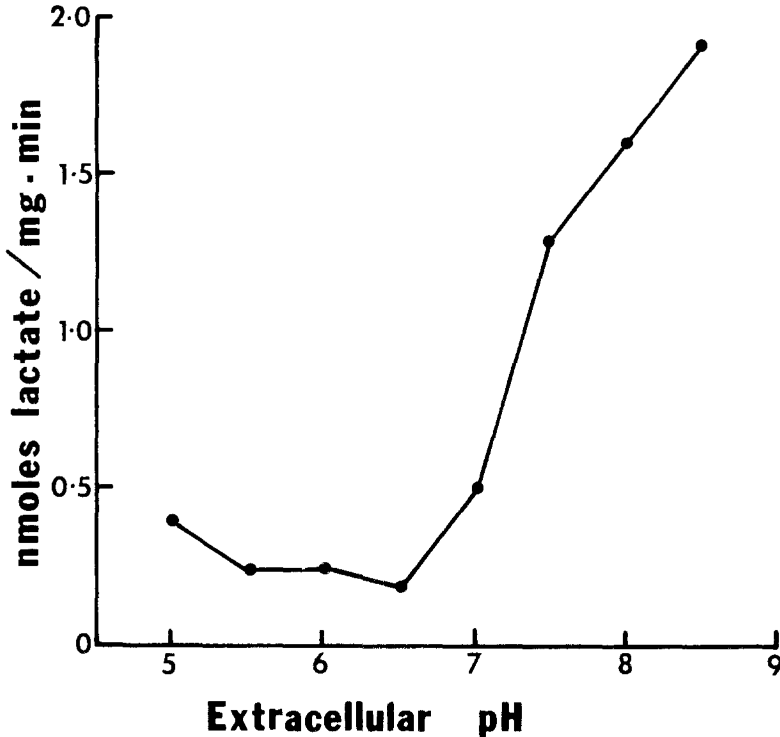

Fig. 2. Dependence of lactate efflux on pH. Lactate efflux from erythrocytes was assayed after 5 min incubation at 20 °C in chloride buffer as described under *Materials and Methods*

Lactate efflux was strongly pH dependent and stimulated with increasing external pH (Fig. 2). In contrast, lactate exchange under equilibrium conditions increased with decreasing pH [3], probably reflecting the direct effect of the pH upon the activity of the transport system. The net flux studies here were done under pH jump conditions and suggest that the pH gradient, rather than absolute pH, was the major factor controlling lactate flux in these experiments.

Lactate Influx

The influx of lactic acid was also pH dependent as shown in Fig. 3. In this case lactate uptake was stimulated at a lower external pH. At pH 6.5 and 7.5 the uptake was a saturable process exhibiting Michaelis-Menten kinetics with a V_{max} of 10 nmol/mg protein · min. The affinity for lactate changed with the external pH with the apparent K_m for lactate

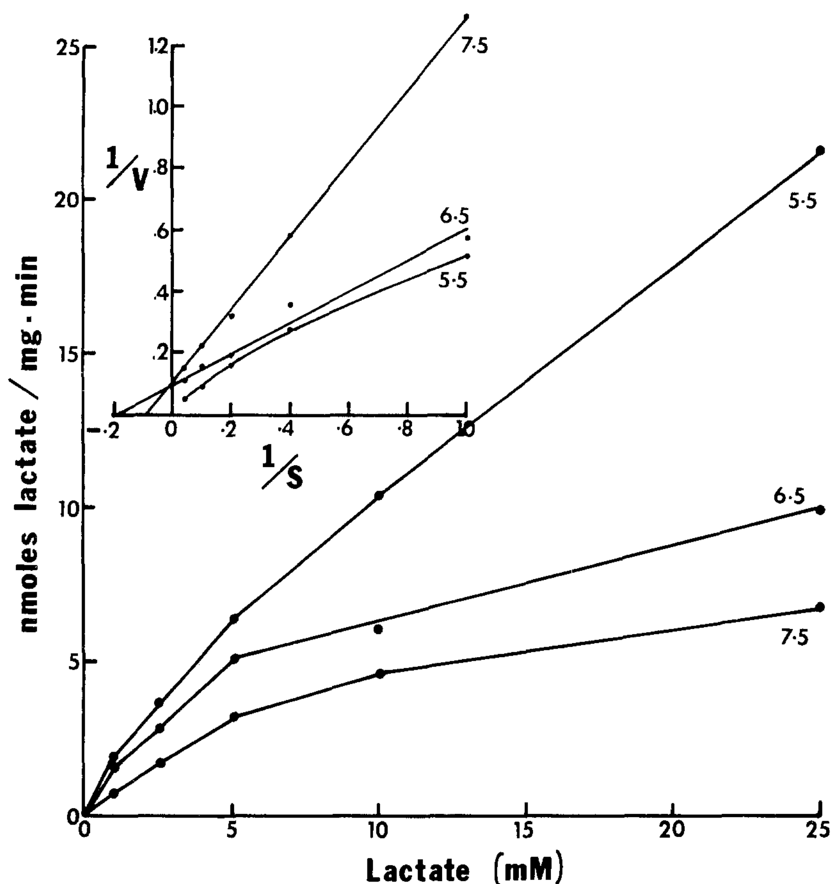

Fig. 3. Dependence of lactate uptake on pH. Lactate uptake into erythrocytes was assayed in 90 mM NaCl plus either 50 mM MES for pH 5.5 and pH 6.5 or 50 mM HEPES for pH 7.5. The concentration of NaCl was adjusted to maintain ionic strength. Cells were incubated for 1 min at 25 °C and processed as described under *Materials and Methods*

decreasing from 13.4 mM at pH 7.5 to 4.5 mM at pH 6.5. At pH 5.5, uptake deviated from saturation kinetics at higher lactate concentrations. This may in part be due to nonionic permeation of lactic acid or the superimposition of a second transport system. The pH dependence of lactate influx and efflux indicates that the direction of the pH gradient across the membrane is the major determinant in the direction of net lactate flux. Two different mechanisms could account for these results. (i) Lactate transport could be driven by a H^+-lactate symport (or OH^-/lactate antiport). (ii) Alternatively [4], a lactate$^-$/Cl^- exchange could be driven by a chloride gradient which is generated by a pH gradient. A change in the medium pH should lead to a redistribution of

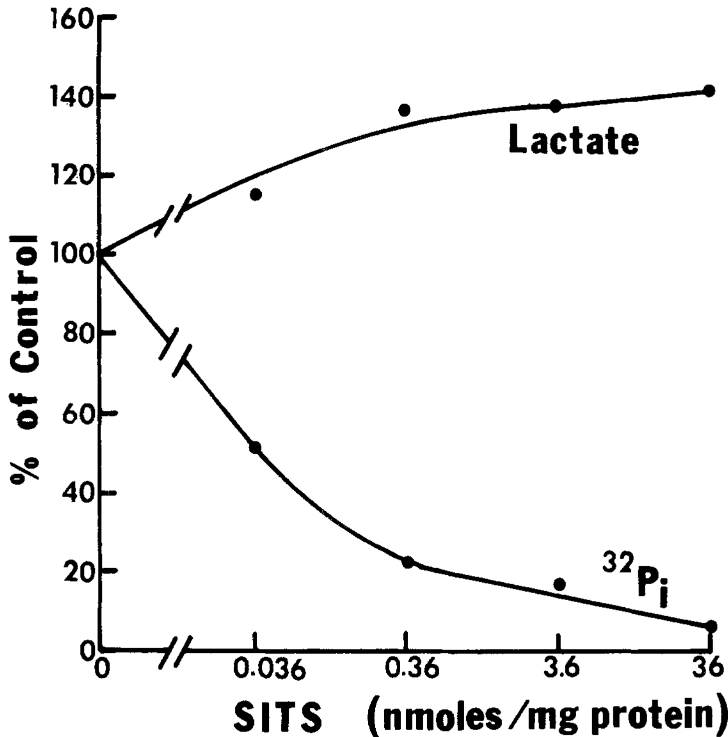

Fig. 4. Effect of SITS on lactate and P_i uptake. Erythrocytes were treated with indicated amounts of SITS for 30 min at 0 °C, then washed three times in chloride buffer. They were then incubated at 25 °C in pH 6.5 buffer, as described in the legend of Fig. 3, with 10 mM lactate or 10 mM Na phosphate

chloride ions across the red cell membrane via the Cl^-/HCO_3^- inorganic anion channel. The latter is SITS-sensitive and responds to an acidification of the medium by an increase in intracellular Cl^-. This basic mechanism would work in either direction of lactate flux since the chloride shift is fully reversible.

Since the direction of Cl^- flux is determined by the relative intra- and extra-cellular concentration of Cl^-, the two mechanisms can be distinguished by varying the Cl^- distribution and by blocking the inorganic anion channel with SITS. Under conditions whereby the anion channel was fully inhibited by SITS as indicated by P_i flux measurements, lactate transport in the presence of external chloride was actually stimulated 30 to 60% (Fig. 4). The possibility that the pH dependence of lactate transport is secondary to a redistribution of chloride is unambiguously ruled out since lactate transport was still pH dependent when Cl^-/HCO_3^- exchange was blocked (Fig. 5).

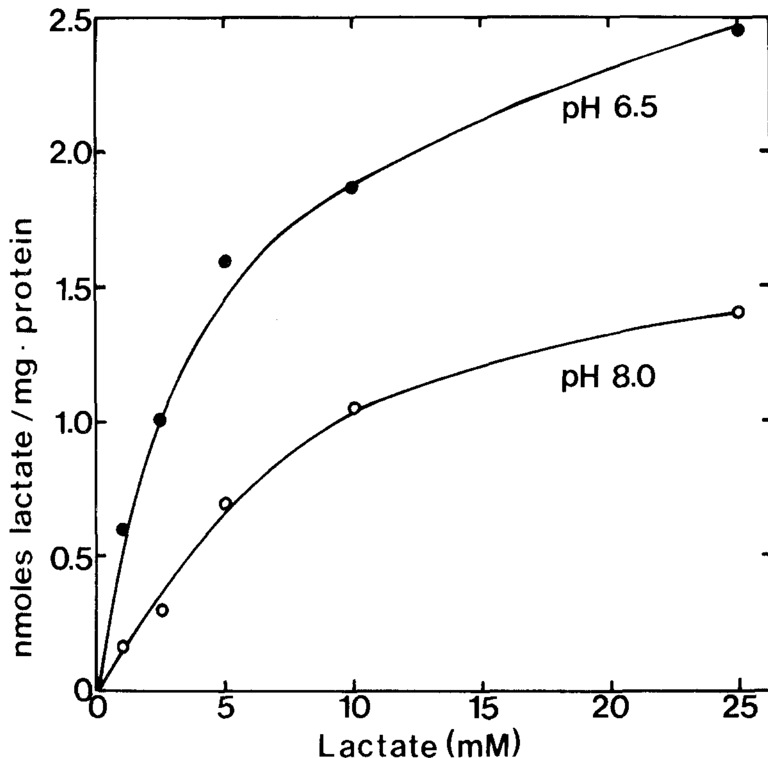

Fig. 5. Dependence of lactate uptake on pH in SITS-treated cells. Cells were treated with 0.5 mM SITS, as described in the legend of Fig. 4, and assayed for lactate uptake for 1 min at 25 °C

The Interaction between Cl^- and Lactate Flux

Since previous investigators have reported inhibition of lactate transport in human red cells by SITS and have used this finding as the strongest evidence for a role of the inorganic anion channel in this process, it was deemed essential to elucidate the condition under which such inhibition can be observed. The SITS-insensitive flux of lactate shown in Fig. 4 provides clear evidence for a distinct monocarboxylate transport system in erythrocytes. When an outwardly directed Cl^- gradient was established by washing erythrocytes that contained Cl^- in Cl^--free buffers, the higher rates of lactate uptake in the absence of chloride (Fig. 6, *compare* to Fig. 3) were suppressed by either extracellular Cl^- or SITS treatment.

These results are readily explained in terms of the two distinct trans-

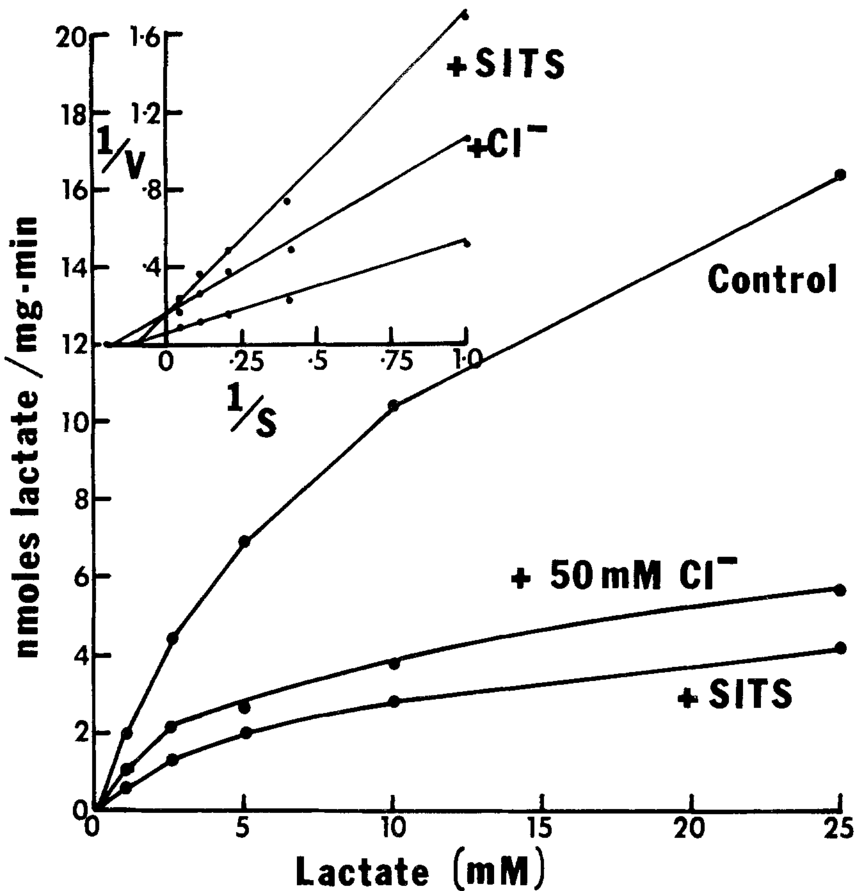

Fig. 6. Effect of chloride and SITS on lactate uptake. Erythrocytes were incubated with chloride buffer for 50 min in the presence or absence of 1 mM SITS at 0 °C. They were then washed three times in sucrose buffer, pH 8, and suspended with an equal volume of sucrose buffer (no chloride). Uptake was assayed in either 200 mM sucrose, 50 mM MES, pH 6.2, or in 50 mM NaCl, 110 mM sucrose and 50 mM MES, pH 6.2, for 1 min at 25 °C

port systems, a H^+-lactate symport, and the inorganic ion channel which allows the exchange of Cl^- against HCO_3^- or OH^- [9]. The latter can create or dissipate a pH gradient across the membrane depending on the direction of flux. External Cl^- moving into the cell dissipates the pH gradient by exchanging for OH^-, diminishing the proton driving force for lactate uptake. An outwardly directed Cl^- gradient increases the pH of the internal compartment, thereby stimulating lactate uptake. In either case the effect of SITS would be to eliminate any contribution of chloride transport to the pH gradient.

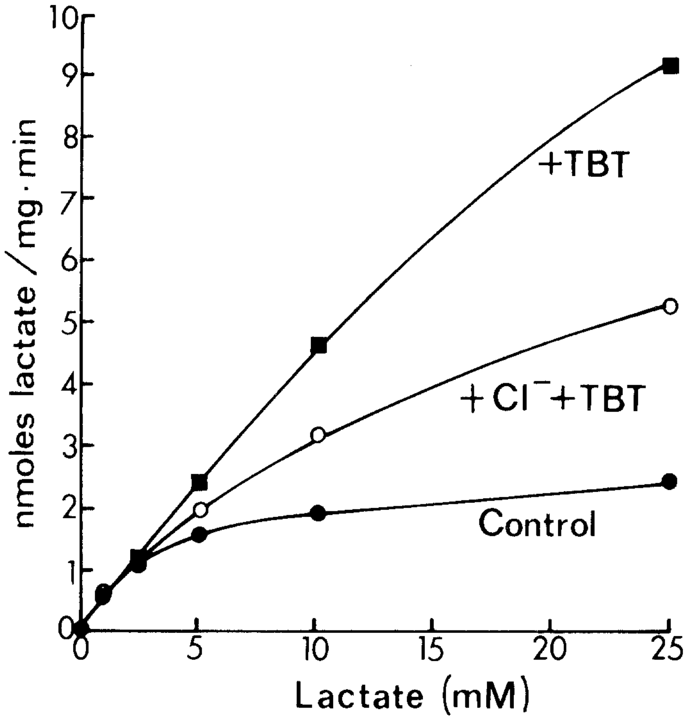

Fig. 7. Tributyltin chloride stimulation of lactate uptake. Cells were pretreated with SITS, washed, and assayed, as described in the legend of Fig. 6. Tri-N-butyltin chloride (5 μM) was added at 0 time

Interaction of Lactate Flux with an Artificial Cl^-/OH^--Exchange

According to the proposed mechanism, chloride flux affects lactate transport indirectly by creating a pH gradient across the membrane. In the presence of SITS, such a gradient can be artificially induced with trialkyltinchloride derivatives, which were shown to act as ionophores for a Cl^-/OH^- antiport in erythrocytes, mitochondria and lipid bilayers [10, 7]. As shown in Fig. 7, after treatment of erythrocytes with SITS, tri-N-butyltin chloride markedly stimulated uptake of lactate over that in control cells when no external chloride was added. This stimulation was suppressed by external chloride, indicating that diminishing the chloride gradient inhibited uptake. It was therefore apparent that the effect of chloride on the lactate transport was indirect – through formation of a proton gradient.

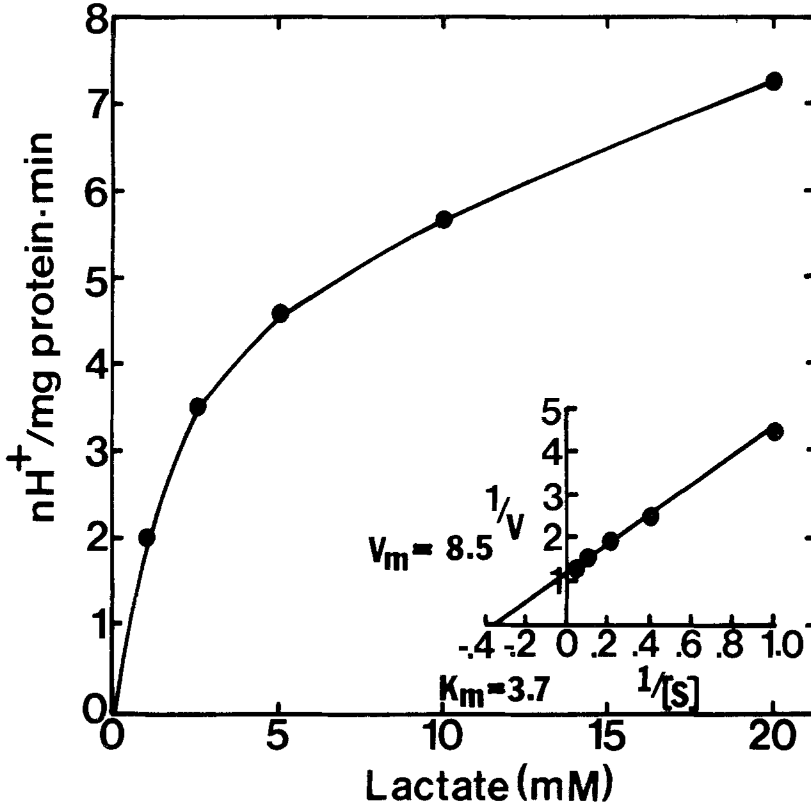

Fig. 8. Lactate dependent proton uptake. Depleted cells were pretreated with 500 μM SITS for 1 hr at 0 °C then washed 3 times in 250 mM sucrose-1 mM Na Tricine, pH 8, 1 mM $MgSO_4$, and suspended with an equal volume of the same buffer. An aliquot of 0.1 ml cells was added to 0.9 ml of 150 mM Na-isethionate, 2 mM $MgSO_5$ and 2 mM NaMES, pH 6.2. After 3–5 min equilibration, sodium lactate, as indicated, was added to initiate proton uptake

H^+ Movements Associated with Lactate Uptake

The major argument against a lactate proton symport mechanism in the human erythrocyte has been the failure to measure proton movements associated with lactate flux [1, 4]. It is necessary to treat the erythrocytes with SITS or DIDS to eliminate chloride flux and thus the drift of pH which was always observed. Moreover, previous studies were done in the absence of a pH gradient. Fig. 8 shows lactate-dependent proton uptake. Uptake was a saturable process and yielded a V_{max} and K_m similar to that determined by ^{14}C-lactic uptake. Direct

measurement of ^{14}C-lactate uptake during the experiment resulted in a H^+/lactate stoichiometry of 0.8 ± 0.1 (18 measurements), with some error due to a variable drift of a basal H^+ uptake by the cells.

All of the results reported in this paper are consistent with a H^+-lactate symport mechanism for lactic acid transport, independent and distinct from the inorganic anion channel, although we cannot rule out that some lactate is transported via this pathway. Qualitatively, the transport system described here is similar to that of ascites tumor cells and *S. faecalis* [5, 11].

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