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DISCRIMINATION OF THREE PARALLEL PATHWAYS OF LACTATE TRANSPORT IN THE HUMAN ERYTHROCYTE MEMBRANE BY INHIBITORS AND KINETIC PROPERTIES

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The transmembrane movements of lactate and other monocarboxylate anions in mammalian erythrocytes have been claimed, by virtue of their sensitivity to SH-reagents, to involve a transfer system different from the classical anion system (Deuticke, B., Rickert, I. and Beyer, E. (1978) Biochim. Biophys. Acta 507, 137–155). Inhibition of monocarboxylate transfer by SH-reagents, however, was incomplete to an extent varying for different monocarboxylates. The transport component insensitive to SH-reagents has now been shown to involve (a) the classical anion-exchange system, as demonstrated by sensitivity to specific disulfonate inhibitors, and (b) nonionic diffusion, as indicated by the characteristic pH- and concentration dependency of this component and its stimulation by aliphatic alcohols. Under physiological conditions about 90% of total lactate movement proceed via the specific system, 5% via the classical anion-transfer system, 5% by nonionic diffusion. These three components of lactate exchange differ in their activation energies. The specific lactate system mediates net fluxes almost as fast as exchange fluxes, in marked contrast to the classical anion-exchange system which mediates halide exchange much faster than halide net movements. The underlying mechanism, for maintenance of electroneutrality, is an OH^--antiport or an H^+-symport as indicated by the particular response of lactate net fluxes to changes of intra- or extracellular pH.

Introduction

The transport of aliphatic monocarboxylate anions, in particular lactate and pyruvate, across cellular and subcellular membranes has been the subject of a number of studies in recent years [1–8]. In case of the erythrocyte [9–14] the characterization of this transport process includes the examination of a possible involvement of the inorganic-anion exchange system, which physiologically catalyses the Cl^--HCO_3^- exchange accompanying CO_2 transport in blood. This exchange system has been well characterized in the last years in terms of its kinetic properties, its inhibitor sensitivities and the molecular identity of the transport protein [15–17]. A comparison of its properties with the transport characteristics of lactate, glycolate and pyruvate has disclosed one major discrepancy which strongly suggests the presence of a separate monocarboxylate transport system: Monocarboxylate transfer is strongly, although

Abbreviations:

DTNB,	5,5′-dithio-bis(2-nitrobenzoate);
DNDS,	4,4′-dinitro-2,2′-stilbenedisulfonic acid;
DIDS,	4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid;
SITS,	4-acetamido-4′-isothiocyano-2,2′-stilbenedisulfonate;
PCMBS,	*p*-chloromercuriphenylsulfonic acid;
8-ANS,	8-anilino-1-naphthalenesulfonic acid;
2,4,6-TCBS,	2,4,6-trichlorobenzenesulfonic acid;
DTDP,	4,4′-dithiodipyridine;
NBD, chloride,	4-chloro-7-nitrobenzo-2,2′-oxa-1,3-diazole;
Hepes,	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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not completely, inhibited by SH-reagents which do not affect the inorganic anion exchange system [11].

Additional evidence in favour of a separate monocarboxylate transfer system came from the observation of a low sensitivity of lactate, pyruvate and β-hydroxybutyrate transfer to stilbene disulfonate derivatives which completely inhibit inorganic-anion transfer at low concentrations [10,11,14]. This low sensitivity and the incomplete inhibition of lactate transfer by SH-reagents suggested a contribution of the inorganic-anion exchange system, which, however, has not been defined as yet in quantitative terms. Very different extents of stilbene disulfonate inhibition of monocarboxylate transfer were reported by different investigators [9,11,14].

A further problem in the analysis of monocarboxylate transfer arises from the possible involvement of nonionic diffusion. It has been demonstrated [18–20] that many aliphatic monocarboxylates permeate the erythrocyte membrane to a major extent by this mechanism. In the experimental assay used for the demonstration of nonionic diffusion lactate and pyruvate behaved as if penetrating only in the ionic form [18]. This assay, however, is of limited value in demonstrating minor contributions of nonionic diffusion in the presence of a large ionic fraction. Moreover, nonionic diffusion of lactate and pyruvate across artificial bilayer membranes has been demonstrated [21,22]. Thus, further information on this problem, based on tracer flux measurements analogous to previous studies [20], appeared to be necessary.

In the following an attempt is presented to quantitatively dissect the transfer of two monocarboxylates, lactate and glycolate, into different components, separable by different inhibitor sensitivities, and characterized by differences in a number of criteria. Preliminary results have been reported elsewhere [23,24].

Materials and Methods

Materials

Human blood was obtained from the local blood bank, anticoagulated with heparin and used for experiments within 24 h after its withdrawal.

Standard chemicals were of the highest purity available. Sodium L-lactate and glycolic acid were obtained from Fluka, Neu-Ulm; L-[U-^{14}C]lactate, [1-^{14}C]glycolate, [U-^{14}C]oxalic acid and sodium [^{35}-S]sulfate from Amersham-Buchler, Braunschweig; 5,5′-dithio-bis(2-nitrobenzoate) (DTNB) and benzene-1,3-disulfonate were purchased from Merck AG., Darmstadt; 4,4′-dinitro-2,2′-stilbenedisulfonic acid, sodium salt (DNDS) from ICN-K and K-Labs Plainview, NY, U.S.A.; *p*-chloromercuriphenylsulfonic acid, sodium salt (PCMBS) and 8-anilino-1-naphthalene sulfonic acid, Mg salt (8-ANS) from Sigma, Munich; sodium tetrathionate, 2,4,6-trichlorobenzenesulfonate (TCBS) and 4,4′-dithiodipyridine (DTDP) from Fluka, Neu-Ulm; 4-chloro-7-nitrobenzo-2,2′-oxa-1,3-diazole (NDB chloride) from Serva, Heidelberg. 4,4′-Diisothiocyano-2,2′-stilbenedisulfonate (DIDS) was a generous gift from Dr. J.O. Wieth, Copenhagen, gramicidin D and niflumic acid were kindly donated by Heyden-Squibb, Regensburg.

Methods

Preparation of erythrocytes, loading of the cells with labelled and unlabelled transport substrates and determination of equilibrium exchange rates from measurements of tracer efflux were carried out as described in a previous paper [11]. Measurements of lactate net efflux were carried out by the same procedure except that the media used for efflux measurements contained neither labelled nor unlabelled transport substrate. Incubation media had the following basic composition (values in mM):

Medium A: NaCl 140; Na_2HPO_4/NaH_2PO_4 6.5; glucose 4.5; lactate (glycolate) 5.4, or sulfate 3.5;

Medium B: KCl 100; NaCl 36; Na_2HPO_4/NaH_2PO_4 6.5; lactate (glycolate) 5.4, or sulfate 3.5; sucrose 27; gramicidin D 5 μg/ml.

Medium A preserves the characteristic distribution of ions. In Medium B cation gradients are abolished due to removal of the cation barrier by gramicidin D. Colloid-osmotic hemolysis is prevented by the extracellular sucrose. This medium was used whenever changes of cation permeability

due to the particular experimental conditions were expected to interfere with efflux measurements, e.g. by changes of membrane potential.

Inhibitors of anion transfer processes were usually added during the period of efflux measurements. In case of DIDS, and sometimes PCMBS also, which are irreversibly or very tightly bound to the membrane, the cells were exposed to the inhibitors already after the loading with the labelled substrate. Subsequently, the erythrocytes were washed one or twice in tracer- and inhibitor-free medium and immediately used for efflux measurements into inhibitor-free media.

For measurements of lactate net efflux at varying external pH, the cells were loaded with the labelled substrate as usual. Efflux was measured into substrate-free media, in which part of the NaCl had been replaced by impermeant or slowly permeating buffers such as phosphate, Hepes and glycylglycine. Details are given in the results.

Results and Discussion

I. Demonstration of a component of lactate transfer resistant to mercurial and dithiol inhibitors

The concept of a particular transfer system for glycolate, lactate, pyruvate and related monocarboxylates in human and other mammalian erythrocytes is predominantly based on the inhibition of transfer by certain SH-reagents, which do not affect inorganic-anion exchange [11]. These inhibitions, however, are incomplete. Their maxima differ for the two anions studied, but are essentially independent of the type of inhibitor, as demonstrated in Fig. 1. Small differences are probably due to secondary phenomena, such as leaks induced by Hg^{2+} [25] and a progressive relief of the inhibition by DTNB at high concentrations of the inhibitor. In case of DTDP, the inhibition of the specific transfer of glycolate was obtained by correcting (see legend to Fig. 1) the inhibition of the total flux of glycolate (inset Fig. 1, closed triangles) for an inhibition of anion flux via the anion exchange system (inset Fig. 1, open triangles). The transfer of anions (e.g. oxalate and sulfate) via the anion exchange system is inhibited by high concentrations of DTDP, but not of the other SH-reagents used here, due to mechanisms presently under investigation.

TABLE I

INFLUENCE OF TWO SH-REAGENTS ON THE EXCHANGE OF ANIONS ACROSS THE HUMAN ERYTHROCYTE MEMBRANE

Cells were loaded with the labelled anions as described in Methods and then exposed to the reagents under the conditions given. After the treatment the cells were washed once, and the back-exchange of tracer measured in the absence of inhibitor. Rate coefficients in arbitrary units relative to the controls.

	Controls	*N*-Ethylmaleimide (10 mM, 90 min, 37°C)	NBD chloride (8 mM, 10 min, 37°C)
Sulfate (35°C)	1.0	0.45	0.32
L-Lactate (20°C)	1.0	0.14	0.09

Other types of SH-modifying agents were not included in our study, since they either do not affect lactate transfer (iodoacetate, iodoacetamide) or inhibit the transfer of both lactate and inorganic anions (cf. Table I). This inhibition, however, is presumably due to an interaction with amino groups [26], since extensive blockage of membrane SH-groups by an SH-specific reagent,

TABLE II

LACK OF INFLUENCE OF A PRETREATMENT OF ERYTHROCYTES WITH 4,4′-DITHIODIPYRIDINE ON THE INHIBITORY EFFECT OF *N*-ETHYLMALEIMIDE ON SULFATE EXCHANGE.

Cells suspended at a Hct of 20% in Medium B without gramicidin were first incubated with or without 5 mM dithiodipyridine at pH 8.5, 37°C for 120 min, then washed three times with saline (pH 7.4) and finally incubated with or without 5 mM *N*-ethylmaleimide at pH 8.0, 37°C for 30 min. After two further washings in saline media, cells were loaded with ^{35}S-sulfate and fluxes measured as described in Methods (pH 7.35, 35°C). Membrane SH-groups determined as described in Ref. 30.

	Rate coefficient (min^{-1})	Membrane SH-groups (nmol/mg protein)
Control	0.0168	75 ± 5
N-Ethylmaleimide	0.0091	26
4,4′-Dithiodipyridine	0.0150	20
4,4′-Dithiodipyridine, then *N*-ethylmaleimide	0.0079	–

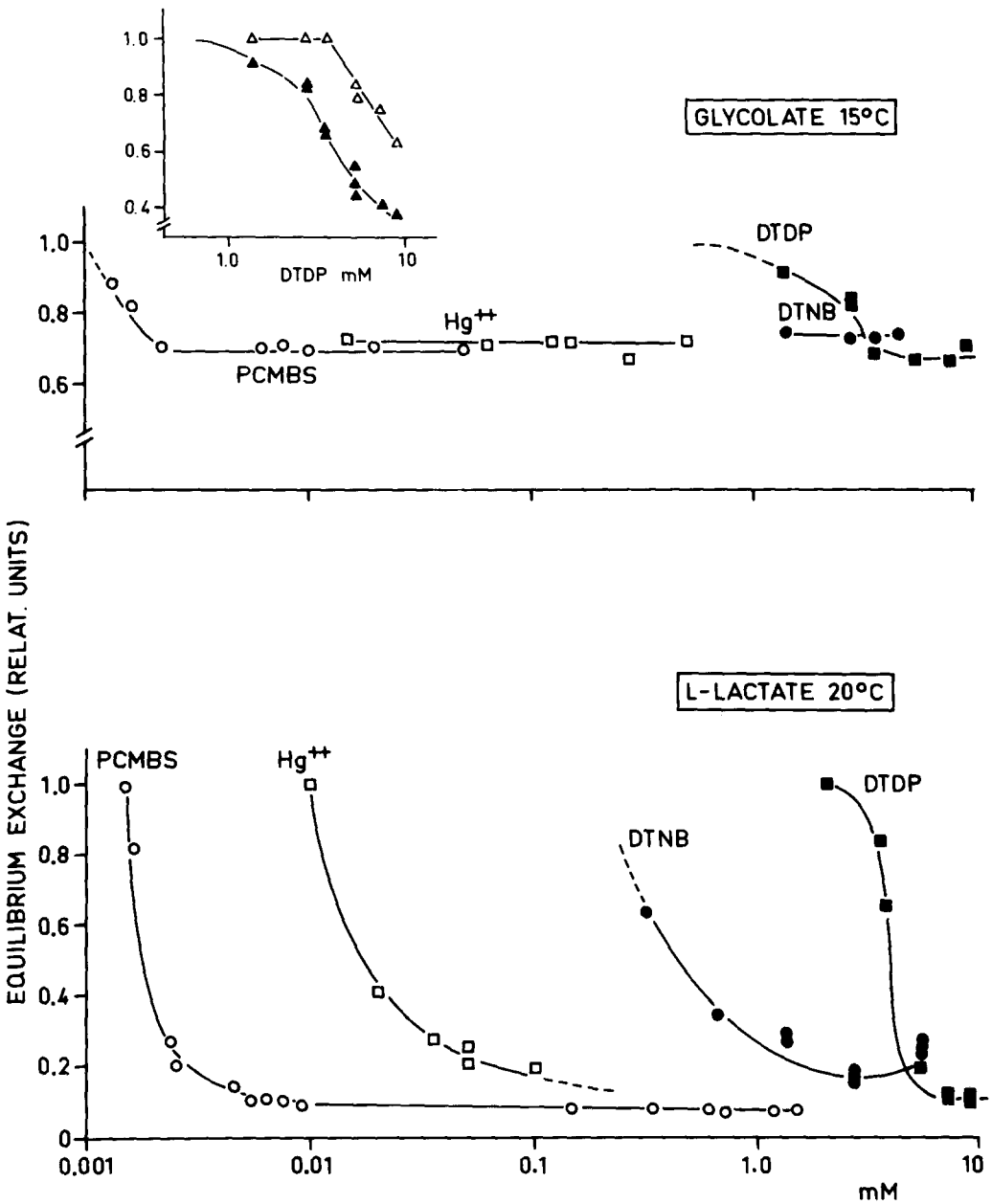

Fig. 1. Incomplete inhibition of L-lactate and glycolate equilibrium exchange by SH-reagents of the mercurial and the dithiol type. Fluxes measured in medium B (pH 7.35, 20°C (L-lactate), 15°C (glycolate)). Exposition of the cells to mercurials during the efflux period, to dithiols during the pretreatment period (before or after loading with labelled test substance). DTDP: 5 min, 37°C, pH 7.4; DTNB: 120 min, 37°C, pH 8.2. Inset: Inhibitory effect of DTDP on total glycolate flux (▲——▲) and on anion movements via the inorganic-anion exchange system, studied by measuring oxalate fluxes (△——△). The decrease of the relative equilibrium exchange of glycolate in the presence of DTDP shown in the main panel was calculated from the curves in the inset by the adding the fractional reduction of oxalate flux (1.0 − relative equilibrium exchange)$_{oxalate}$ at a given concentration of DTDP to the relative equilibrium flux of glycolate at this concentration. By this correction one obtains the selective effect of DTDP on transport of glycolate via the specific monocarboxylate system.

TABLE III

CONTRIBUTION OF THE PCMBS-INSENSITIVE FRACTION OF L-LACTATE EQUILIBRIUM EXCHANGE TO THE TOTAL EXCHANGE AT DIFFERENT CONCENTRATIONS OF L-LACTATE

Fluxes were measured in anisotonic media containing besides L-lactate at the concentrations given only Na_2HPO_4/NaH_2PO_4 6.5 mM, sucrose 27 mM and gramicidin D 5 μg/ml (20°C, pH 7.4). Cells were pretreated with PCMBS and fluxes measured in the absence of the reagent.

Extracellular L-lactate (mM)	Exchange flux		
	Control (pmol · $cm^{-2} \cdot s^{-1}$	0.6 mM PCMBS	
		pmol · $cm^{-2} \cdot s^{-1}$	% of control
1.5	0.13	0.007	5.4
46	4.0	0.36	9.0
267	7.6	2.5	32.0

4,4'-dithiodipyridine, prior to the exposure to *N*-ethylmaleimide does not preclude inhibition (Table II) *.

The identical inhibitions obtained with mercurials and dithiols justify the conclusion that (at 20°C, pH 7.4 and an extracellular substrate concentration of 5 mM) only about 6% of the self-exchange of L-lactate, but 60–70% of the glycolate exchange, proceed by a pathway differing from the SH-dependent route.

In case of L-lactate the contribution of the SH-independent pathway is small under the conditions mentioned above. It increases, however, under a number of circumstances, e.g. with temperature, at low pH and, particularly, at high lactate concentrations (Table III). From this latter observation it follows that the concentration dependencies of the SH-dependent and the SH-independent transfer of L-lactate must be different. The SH-dependent pathway exhibits saturation (cf. also Refs. 10, 11 and 23) in contrast to the SH-independent one.

* Inhibition of the inorganic-anion exchange system by DTDP is only observed when cells are exposed to the agent for short periods and not washed free of reagents afterwards. Prolonged exposure to the agent and subsequent washing abolish inhibition.

II. Characterization of the lactate transfer resistant to mercurials

(a) Disulfonate-sensitive transfer

The most likely basis of an SH-independent monocarboxylate exchange are movements via the inorganic-anion exchange system [9,10,11]. To confirm this assumption, inhibitors of inorganic-anion exchange were used. Reversible amphiphilic inhibitors such as phloretin, salicylate or tetracaine [27], as well as 8-ANS [28], 2,4,6-TCBS [11] and niflumic acid [29] inhibit lactate transfer to the same extent as anion exchange via the inorganic-anion exchange system (Fig. 2). Inhibition of lactate transfer by these compounds must result from an effect on the PCMBS-sensitive fraction of monocarboxylate transfer, since it exceeds the PCMBS-insensitive fraction (approx. 5–10%) of the transfer. The same is true for covalently bound inhibitors such as fluorodinitrobenzene [11] or pyridoxal phosphate (unpublished results). The very low sensitivity of lactate exchange to disulfonates (SITS, tetrathionate) [11,14], on the other hand, suggested specificity of these inhibitors for the inorganic-anion exchange system. This specificity was substantiated in experiments with different disulfonate inhibitors bound either non-covalently (tetrathionate [30], DNDS [15]) or covalently (DIDS [15]). At concentrations completely inhibiting the transfer of sulfate (or oxalate), these agents reduce the exchange of lactate and glycolate only to an extent which roughly corresponds to the fraction insensitive to SH-reagents (Fig. 3). At very high concentrations, however, the inhibition increases progressively (data not shown). Obviously,

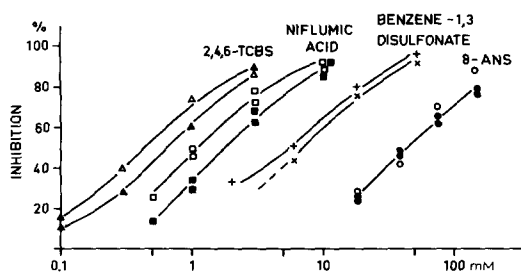

Fig. 2. Dose-response curves for the inhibition of L-lactate (▲, ■, ×, ●) and oxalate (△, □, +, ○) equilibrium exchange by various amphiphilic inhibitors. Inhibitors present during the efflux period, fluxes measured in medium A (pH 7.35, 10°C, Hct 5%).

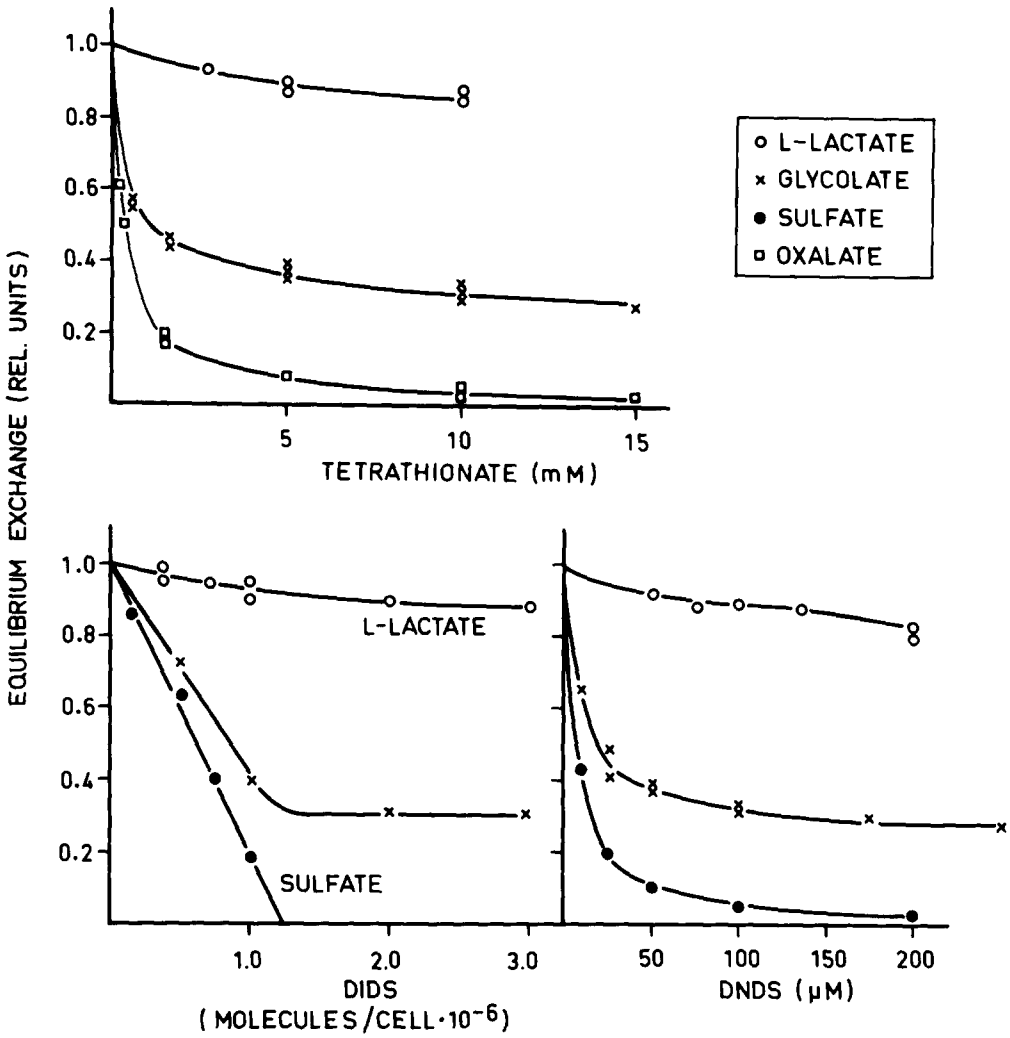

Fig. 3. Inhibition of L-lactate, glycolate, sulfate, and oxalate equilibrium exchange by disulfonate inhibitors. Fluxes measured in medium A, pH 7.35, L-lactate 20°C, glycolate 15°C, sulfate 37°C, oxalate 10°C. Tetrathionate and DNDS were present during the efflux period. Exposure to DIDS during a 45-min treatment of the cells at 37°C before the efflux measurements.

these agents also inhibit monocarboxylate transfer, but with a very low effectivity. It was therefore difficult to decide whether or not the partial inhibition observed at low concentration could serve as a quantitative indicator of lactate transfer via the inorganic anion exchange system.

In order to obtain more reliable information on this point, the effects of disulfonates on lactate or glycolate transfer were studied after blockage of the PCMBS-sensitive component. The PCMBS-insensitive components of L-lactate and glycolate transfer are inhibited much more effectively by disulfonates than the total transfer of these two anions. In case of glycolate (Fig. 4, right panel), the resulting dose-response curves (at 15°C) essentially coincide with those for sulfate or oxalate*, indicating that the PCMBS-insensitive glycolate movements occur via the inorganic-anion exchange pathway. Only at 30°C (DIDS experi-

* Oxalate fluxes can be measured at 10°C. Oxalate was therefore used as a test anion in studies with tetrathionate, since dose-response curves for inhibition of anion exchange by tetrathionate are highly temperature-dependent [30].

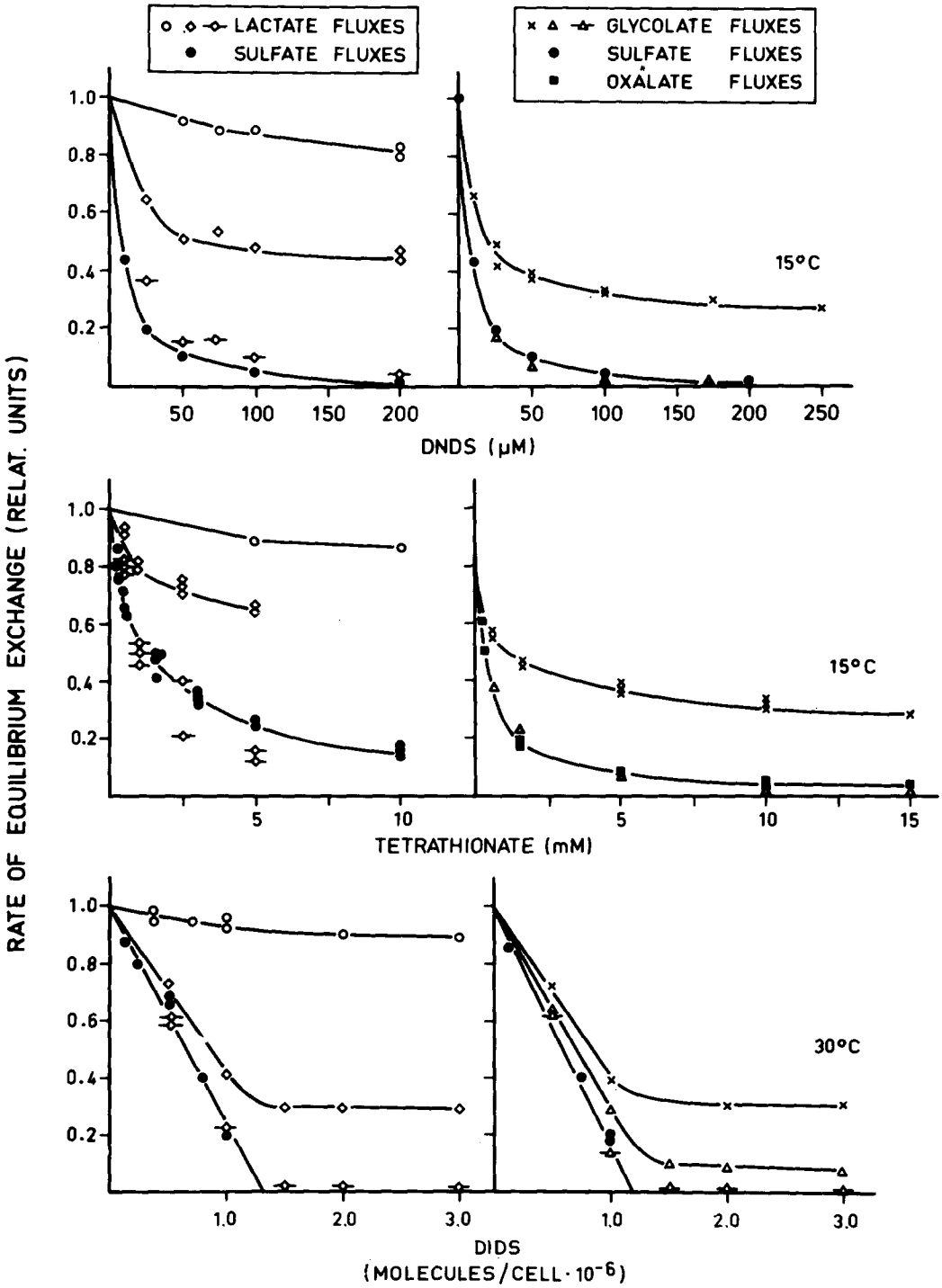

Fig. 4. Dose-response curves for the inhibition by disulfonate inhibitors of the equilibrium exchange of L-lactate (left panel) and glycolate (right panel). Curves for the inhibition of sulfate (●) and oxalate (■) equilibrium exchange are given for comparison. Fluxes were measured in medium B (at pH 7.35 and the following temperatures: L-lactate: 30°C, glycolate: 15°C, 30°C (DIDS-experiments), sulfate: 35°C, oxalate: 10°C). ○, ×, ●, ■: Fluxes of the four anions at different disulfonate concentrations, relative to fluxes without any inhibitor (= disulfonate-sensitive fluxes). ◇, △: Fluxes in presence of PCMBS (0.6 mM) plus disulfonate inhibitors, relative to fluxes in presence of PCMBS (0.6 mM) only (= disulfonate-sensitive, PCMBS-insensitive fluxes). -◇-, -△- : Fluxes obtained as above in presence of PCMBS and disulfonate, but after correction of the values for the fraction of the total flux insensitive to both types of inhibitors. Correction achieved by subtracting the rate coefficient of the residual flux, insensitive to saturating concentrations of both inhibitors, from the rate coefficients for both, the PCMBS-insensitive flux component, and the flux measured, in presence of PCMBS, at the different (submaximally effective) concentrations of each disulfonate. Note the coincidence between these corrected dose-response curves and those for sulfate or oxalate exchange.

TABLE IV

AGREEMENT BETWEEN THE RATES OF L-LACTATE TRANSFER INSENSITIVE TO PCMBS + DISULFONATES AND TO AMPIPHILIC INHIBITORS

Fluxes measured in medium A, at 30°C, pH 7.35, in the presence of the inhibitors, except in case of DIDS, which was applied during a preincubation period (30 min, 37°C).

	k (min^{-1})
PCMBS (0.3 mM) + DIDS (50 nmol/ml cells)	0.0205
PCMBS (0.3 mM) + DNDS (0.15 mM)	0.0228
PCMBS (0.3 mM) + tetrathionate (5 mM)	0.0225
Salicylate (20 mM)	0.0233
Phloretin (0.25 mM)	0.0243

ments) an inhibitor-insensitive fraction became detectable.

Dose-response curves for the fractional inhibition of the PCMBS-insensitive component of lactate transfer (Fig. 4, left panel) by disulfonates differ from those for sulfate or oxalate transfer [30]*. A residual fraction of lactate transfer is insensitive to both, mercurial and disulfonate inhibitors. This residual transfer corresponds to the transfer rates of lactate observed in cells exposed to maximally effective concentrations of amphiphilic inhibitors such as salicylate or phloretin, which affect both anion transfer systems (Table IV). In view of this quantitative agreement it seemed justified to postulate that the fraction of L-lactate transfer resistant to all inhibitors represents a third, independent pathway. Dose-response curves were therefore constructed for disulfonate inhibition of PCMBS-insensitive lactate transfer after correction for the rate coefficient of the fraction insensitive to all inhibitors (Fig. 4). The resulting curves well coincide with dose response curves for the inhibition of sulfate (or oxalate) transfer (Fig. 4). This agreement provides evidence that the PCMBS-insensitive, disulfonate-sensitive fraction of lactate transfer proceeds via the inorganic-anion exchange system.

This latter system does not distinguish between the L- and the D-isomer of lactate, as becomes evident from a comparison of the PCMBS-insensitive components of the self-exchange of L- and D-lactate (Table V, (2) − (3)). The rate coefficients for these components of the flux essentially agree for the two isomers (0.0311 versus 0.0280 min^{-1}). Further evidence for the claim that the inorganic-anion exchange system is not stereoselective can be derived from our previous observation that in animals which lack a PCMBS-sensitive lactate exchange (ox, sheep) no selectivity between L- and D-lactate exists [11], while transport is highly, although not completely, sensitive to disulfonate inhibitors (Deuticke, B., unpublished data).

(b) Inhibitor-insensitive transfer

Nonionic diffusion of lactate has been demonstrated in artificial lipid membranes [21,22] and therefore seemed a likely basis for the inhibitor-insensitive fraction of lactate transfer. In order to test this assumption, the pH dependency of the inhibitor-insensitive fraction and its sensitivity to a small aliphatic alcohol were studied. Anion transfer by nonionic diffusion by virtue of its mechanism increases linearly with the concentration of the undissociated acid. Lowering of pH in a range well above the pK' of the acid should therefore lead to an exponential increase of the rate of exchange. Lactate fluxes were measured in cells

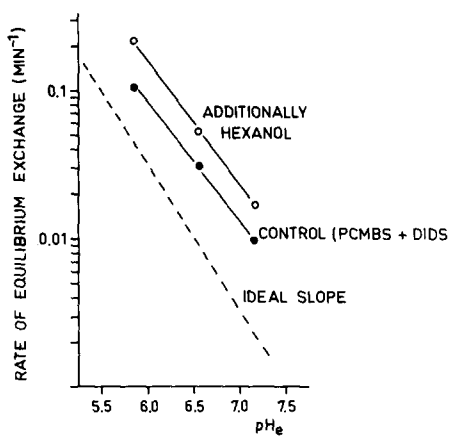

Fig. 5. pH dependency and stimulation by hexanol of the residual L-lactate exchange in presence of disulfonate and PCMBS. O——O, cells, suspended in phosphate-buffered saline were first exposed to DIDS ($3.5 \cdot 10^6$ molecules per cell, 30 min, 37°C and than to PCMBS (1 mM, 5 min, 37°C). After thorough wasing, fluxes were measured (in medium B) at 25°C as described in the methods. The efflux medium contained 0.15 mM PCMBS. ●——●: cells pretreated as above, efflux medium containing hexanol 8 mM.

* See footnote on p. 101.

TABLE V

CONTRIBUTION OF THE THREE COMPONENTS OF TRANSFER TO THE TOTAL EXCHANGE OF LACTATE AND GLYCOLATE THROUGH THE HUMAN ERYTHROCYTE MEMBRANE

Transfer of L- and D-lactate measured in Medium A at 30°C, glycolate in Medium B at 15°C.

	(1) Total exchange k (min^{-1})	(1)−(2) Specific system		(2) PCMBS-insensitive k (min^{-1})	(2)−(3) Inorganic-anion exchange system		(3) (PCMBS-+ disulfonate)-insensitive k (min^{-1})	(3) Nonionic diffusion	
		min^{-1}	%		min^{-1}	%		min^{-1}	%
L-Lactate (30°C)	0.5500	0.4968	90.3	0.0532	0.0311	5.7	0.0221	0.0221	4.0
D-Lactate (30°C)	0.0916	0.0496	54.1	0.0420	0.0280	30.5	0.0140	0.0140	15.5
Glycolate (15°C)	0.0703	0.0238	33.8	0.0469	0.0469	66.2	–	–	–

exposed to PCMBS plus disulfonate. The rate coefficient of the residual flux in fact increased exponentially with lowering of extracellular pH (Fig. 5). The slope of the relationship between pH and log k differs somewhat from the theoretical value of -1 (= 10-fold change of flux per pH unit) but agrees with the slopes observed for the transfer of salicylate or acetate, anions penetrating almost exclusively by nonionic diffusion [20,31]. The deviation is probably due to the fact that intracellular pH, which determines the concentration of intracellular undissociated lactic acid, only changes by 0.8–0.9 units (depending on the range of pH) per 1.0 unit of extracellular change of pH in freshly drawn blood [32]. The pH dependency is therefore reconcilable with nonionic diffusion of lactate through the lipid domain of the erythrocyte membrane.

This assumption was further supported by a marked acceleration of the inhibitor-insensitive lactate transfer in the presence of hexanol (Fig. 5). Small aliphatic alcohols accelerate nonelectrolyte diffusion via artificial lipid bilayers and via the lipid domain of biomembranes, while inhibiting protein-mediated transports [27]. The acceleration of the inhibitor-insensitive fraction of lactate transfer by hexanol therefore indicates diffusion via the lipid domain, almost certainly by nonionic diffusion, in line with observations on acetate transfer [27].

Hexanol can also be used as an instrument for demonstrating different pathways of lactate transfer: In the absence of other inhibitors lactate exchange is blocked by the alcohol like the exchange of sulfate (Fig. 6). This inhibition, however, is only observed at pH 7.4. At pH 5.8 lactate exchange is accelerated by hexanol. This shift from inhibition to acceleration may be interpreted by an increase,

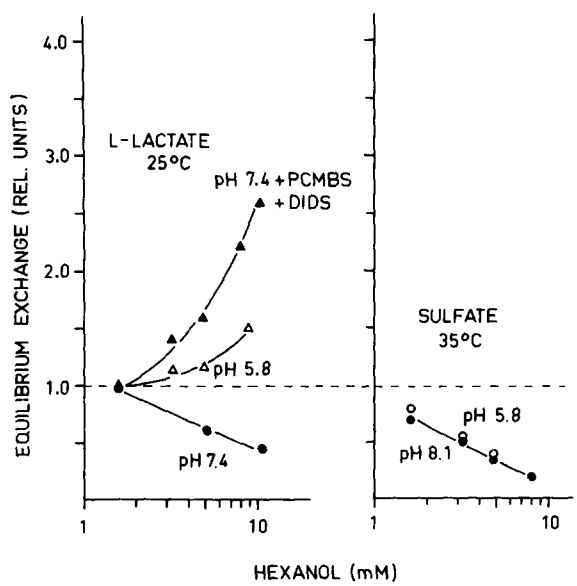

Fig. 6. Influence of hexanol on the equilibrium exchange of L-lactate and sulfate at different pH values and in the presence of inhibitors. Fluxes measured in medium B, hexanol present only during efflux. Pretreatment with DIDS and PCMBS as described in Fig. 5.

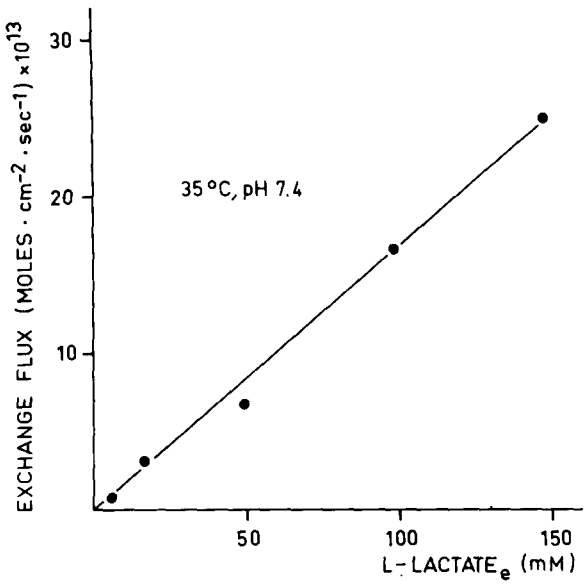

Fig. 7. Linear dependency of the inhibitor-insensitive equilibrium exchange fluxes of L-lactate on lactate concentration. Cells pretreated with DIDS ($2 \cdot 10^6$ molecules per cell) and PCMBS (0.9 mM) as described in Fig. 5. Lactate concentration was increased in isosmolar exchange for chloride.

at low pH, of the contribution of the hexanol-stimulated (= nonionic) component of lactate exchange at the expense of the two hexanol-inhibitable (= ionic) components of lactate transfer.

A final point of evidence for the nonionic nature of the PCMBS-plus-disulfonate-insensitive lactate exchange is given in Fig. 7, which demonstrates a linear relationship between lactate concentration and lactate exchange up to concentrations of 150 mM lactate. These simple diffusion kinetics differ from the saturation kinetics expected and observed in the case of the mediated exchange of lactate [10,11].

III. Features of the three parallel pathways of lactate transfer

The data presented above strongly suggest that the pathways of monocarboxylate transfer defined on an operational basis by inhibitor sensitivities in fact represent structurally different transport routes in the erythrocyte membrane. Crucial evidence for this concept comes from the quantitative agreement of the maxima of inhibition produced by different classes of inhibitors. If these maxima, i.e. the partial insensitivity to inhibitors, were due to incomplete inhibition of one single pathway, as was demonstrated for the effect of certain disulfonate inhibitors on inorganic anion exchange [33], an agreement between chemically different inhibitors would be rather unlikely.

The lack of effect of stilbene sulfonates and of tetrathionate on lactate transfer clearly distinguishes these agents from all other inhibitors of anion movements across the erythrocyte membrane. All other agents tested so far inhibit both, the inorganic-anion exchange and the lactate transfer system, to almost the same extent. This quantitative agreement points to a low specificity of the inhibitors. Inhibition might thus be due to indirect effects such as changes of surface charge densities, of the dipole potential at the interface, or of the packing density and rigidity of membrane lipids. All these parameters can be affected by the amphiphilic compounds [34,35] and were claimed to be involved in the effects of inhibitors of anion transport [28,36–38]. Alternatively, the inhibitors might be bound to sites having a very similar topography on the two transport proteins. The lack of effect of the stilbene disulfonates on

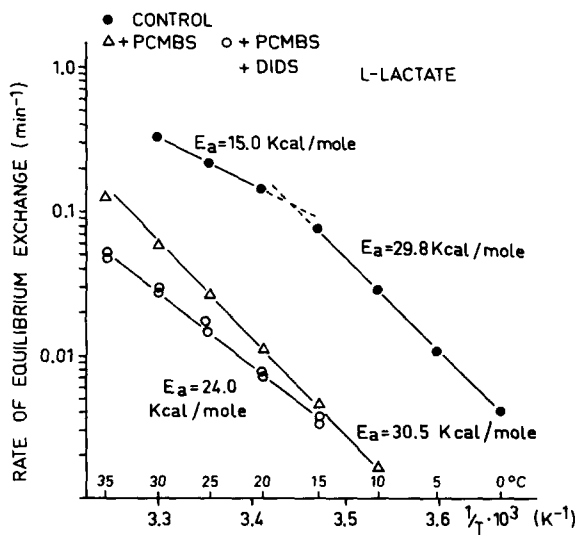

Fig. 8. Arrhenius diagrams for L-lactate equilibrium exchange. ●——●, Fluxes measured in medium A; △——△, fluxes measured in medium A, 0.5 mM PCMBS present during efflux; ○——○, cells pretreated with DIDS ($2 \cdot 10^6$ molecules per cell) and PCMBS 0.9 mM as described in Fig. 5. Fluxes measured in medium A.

lactate movements would then simply result from the inability of the transfer system to accommodate two sulfonate groups at a fairly large distance, since monosulfonates (8-ANS, and 2,4,6-TCBS) and even a disulfonate with its two SO_3^--groups located more closely to each other (1,3-benzenedisulfonate) act equally inhibitory on both transfer systems. Reasons for the specificity of tetrathionate will be dealt with below.

As shown above, SH-reagents and disulfonate inhibitors allow a quantitative dissection of the three pathways of L-lactate transfer by stepwise inhibition. This dissection will in turn provide the corrections required for further quantitative studies on the properties and kinetics of the single components of lactate transfer. As yet, the fractional contribution of the three components could not be assessed directly for normal in vivo conditions (low (< 5 mM) lactate concentration, pH 7.3–7.5, 37°C), since total lactate exchange is too fast here for measurements by conventional techniques. Data for 30°C are given in Table V. The fractional contributions of both, lactate exchange via the inorganic-anion exchange system and by nonionic diffusion, are low. They increase, however, with temperature as a result of different activation energies (Fig. 8) for the three processes.

Total lactate exchange has an apparent activation energy (E_a) of only 15 kcal at temperatures above 15°C. PCMBS-insensitive, disulfonate-sensitive transfer, in contrast, is characterized by a value of 29 kcal/mol, in agreement with data for other anions passing via the inorganic-anion exchange system. Nonionic transfer of lactate has an activation energy of 24 kcal/mol, to be compared with values for other nonionic diffusion processes or non-mediated nonelectrolyte transfer through the erythrocyte membrane (acetate 24 [29], salicylate 13 [31], glycerol 20, erythritol 24–25 kcal/mol [11]).

By extrapolating these Arrhenius plots to 37°C one obtains for L-lactate a fractional contribution of the specific lactate carrier of about 90%. The inorganic-anion exchange system accounts for about 6%, and nonionic diffusion for 4% of the total transfer. Lowering of pH and increasing lactate concentration will change these relationships in favour of the two non-specific pathways. Quite different relationships will also prevail in the case of monocarboxylates with a lower affinity for the specific system (e.g. D-lactate); or with a high lipid solubility and/or a higher pK_a value, properties which favour nonionic diffusion. Kinetic studies on β-hydroxybutyrate transport, a process probably involving the monocarboxylate system [12,13], may require corrections for this fraction.

From the rate coefficient for the transfer of L-lactate by nonionic diffusion the membrane permeability to L-lactic acid can be computed. The calculation is based on the consideration that in case of nonionic diffusion the total equilibrium exchange of lactate ($J_{\bar{A}}$) occurs by movements of the minute fraction of lactic acid (J_{AH}), i.e.

$$J_{\bar{A}} = J_{AH} \tag{1}$$

This means that we can calculate the permeability (P_{AH}) of AH from $J_{\bar{A}}$ and C_{AH} according to

$$P_{AH} = J_{\bar{A}} / C_{AH} \tag{2}$$

$J_{\bar{A}}$ can be obtained by

$$J_{\bar{A}} = k_{\bar{A}} \cdot C_{\bar{A}_i} \cdot \frac{V_i}{F} \tag{3}$$

where $k_{\bar{A}}$ is the measured rate coefficient of tracer exchange of 'lactate', $C_{\bar{A}_i}$ the intracellular concentration of lactate (dissociated and undissociated) and V_i and F the aqueous space and the surface area of the erythrocyte. From (2) and (3) it follows that

$$P_{AH} = k_{\bar{A}} \cdot \frac{C_{\bar{A}_i}}{C_{AH}} \cdot \frac{V_i}{F} \tag{4}$$

From the Henderson-Hasselbalch relationship we obtain

$$\frac{C_{\bar{A}_i}}{C_{AH}} = 1 + 10^{pH_i - pK_A} \tag{5}$$

and thus

$$P_{AH} = k_{\bar{A}} \cdot (1 + 10^{pH_i - pK_A}) \cdot \frac{V_i}{F} \tag{6}$$

This procedure allows determinations of extremely high permeabilities without the necessity of taking

TABLE VI

COMPARISON BETWEEN PERMEABILITIES OF THE HUMAN ERYTHROCYTE MEMBRANE AND BLACK LIPID MEMBRANES (EGG PHOSPHATIDYLCHOLINE IN *n*-DECANE) TO LACTIC AND ACETIC ACID

Data for erythrocytes determined as described in the text, data for black lipid membranes from Ref. 22.

	Erythrocyte membrane		Black lipid membrane P_{AH} ($cm \cdot s^{-1}$)
	$k_{\tilde{A}}$ (min^{-1})	P_{AH} ($cm \cdot s^{-1}$)	
L-Lactic acid ($pH_i = 7.29$)			
30°C	0.0221	$3.7 \cdot 10^{5}$	$5 \cdot 10^{-5}$ (25°C)
Acetic acid ($pH_i = 7.35$)			
0°C	0.1490	$3.4 \cdot 10^{-5}$	
30°C *		$2.3 \cdot 10^{-3}$	$2.4 \cdot 10^{-4}$ (25°C)

* Calculated from values measured at 0°C using the activation enthalpy $E_a = 23$ kcal/mol [27].

into acount unstirred-layer effects [39].

Values obtained for L-lactic acid from the present data and for acetic acid from earlier measurements (Ref. 20, and unpublished data) are compiled in Table VI and compared with data reported for the permeability to these two acids in black lipid membranes from egg phosphatidylcholine in *n*-decane [22]. In the case of lactic acid the data compare well to each other, whereas in the case of acetic acid the erythrocyte membrane seems to be much more permeable than the artificial membrane. Accepting these data one may conclude that the complex lipid domain of the erythrocyte membrane is more selective towards solutes than the simple artificial system, a notion also borne out by other data [27].

IV. Characteristics of SH-dependent lactate transfer

The properties of the specific monocarboxylate carrier can now be studied in the absence of direct and, more important, indirect interference of the inorganic-anion exchange system, due to its insensitivity to disulfonate inhibitors. Taking advantage of this possibility we investigated a particular detail of the lactate transfer system, namely its affinity for inorganic anions. Our studies were prompted by the observation that lactate efflux into lactate-free media occurs at a rate about 2/3 that of lactate efflux into media containing 5 mM lactate. The most simple interpretation, that in the lactate-free media Cl^- acts as an exchange partner for lactate, had to be abandoned in view of the finding (Table VII) that the rate of lactate efflux into lactate-free media is the same in the absence and the presence of Cl^-, or sulfate. These experiments could be carried out under strictly comparable conditions, with respect to pH and intracellular anion concentrations, due to the application of DIDS, which prevented anion movements via the inorganic anion exchange system including those responsible for the extracellular pH shifts occurring when erythrocytes are suspended in weakly buffered solutions of impermeant anions or of nonelectrolytes [40].

A lack of influence of chloride on lactate movements, in contrast to a pronounced effect on sulfate transfer, could also be demonstrated by studying lactate equilibrium exchange (Fig. 9). This chloride insensitivity provides further evidence for the

TABLE VII

LACK OF EFFECT OF EXTRACELLULAR CHLORIDE (OR SULFATE) ON EFFLUX OF L-LACTATE

Cells pretreated with DIDS ($3 \cdot 10^6$ molecules/cell) $pH_i = 7.3$, $pH_e = 8.00$, 10°C, $Lac_i = 3.8$ mM.

Extracellular anions	Rate coefficient of L-lactate efflux (min^{-1})
Chloride 135 mM + L-lactate 5.4 mM	0.0670
Chloride 135 mM	0.0417
Chloride < 1 mM *	0.0449
Sulfate 100 mM	0.0446

* Isotonicity maintained by sucrose.

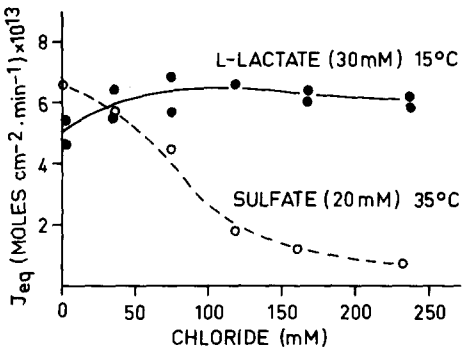

Fig. 9. Different response of L-lactate and sulfate self-exchange fluxes to variation of the chloride concentration. Cells were incubated and loaded with labelled substrate in anisotonic media containing besides NaCl at the concentrations given, Na_2HPO_4/NaH_2PO_4 6.25 mM, sucrose 44 mM, gramicidin D 5 μg/ml, L-lactate 30 mM or sulfate 20 mM. Equilibrium-exchange fluxes were calculated from the measured rate coefficients of tracer efflux, the water content of the cells and the Donnan distribution of lactate or sulfate, as derived from the isotope distribution between cells and medium after establishment of tracer equilibrium.

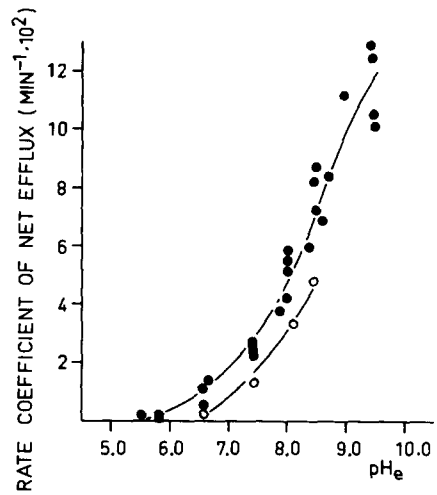

Fig. 10. Dependency of L-lactate net efflux on extracellular pH. Erythrocytes were loaded with unlabelled and labelled L-lactate in medium A (lactate concentration 5.5 mM, pH 7.34 or 7.95.) After one washing at 0°C the cells were suspended in media of varying pH, corresponding to medium A, but free of L-lactate and containing either Na_2HPO_4/NaH_2PO_4 or Na_2HPO_4 30 mM, Hepes 25 mM, and glycylglycine 30 mM in partial replacement for NaCl. Cells were either pretreated with DIDS ($3 \cdot 10^6$ molecules per cell, 30 min, 37°C) or exposed to DNDS (150 μM) during the efflux period, in order to block inorganic-anion exchange and thus pH equilibration. Net efflux of lactate was determined by measuring tracer efflux. Data points obtained before 20% of the radioactivity had left the cells could be fitted to first-order kinetics and were evaluated by the procedure used for equilibrium-exchange studies [11]. Intracellular pH values and lactate concentrations calculated from extracellular pH and lactate during the loading period at 37°C, using appropriate Donnan coefficients. ●, pH_i 7.25; ○, pH_i 7.95.

essential dissimilarities between the transfer system for lactate and for inorganic anions and indicates that the former system does not transport chloride. In addition, the Cl^- insensitivity of lactate transfer may also provide an interpretation for the lack of inhibition of the specific lactate system by tetrathionate: in spite of its high inhibitory potency this anion seems to interact with binding sites for inorganic anions on the inorganic -anion exchange system [30].

Our observation of a rapid lactate efflux into media free of lactate and other anions finally raises the question of the nature of that process. Dubinsky and Racker [14] have reported synchronous movements of H^+ (or OH^-) and lactate across the human erythrocyte membrane, indicating the presence of either a lactate-H^+ cotransport or a lactate/OH^- antiport system. This concept is further borne out by experiments in which we studied the net efflux of lactate into lactate-free media of widely varying pH, at constant intracellular pH. pH equilibration via the inorganic-anion exchange system was essentially prevented by pretreatment of the cells with DIDS or addition of DNDS to the efflux media and adequate buffering of the external media. As shown in Fig. 10, rates of lactate net efflux increase progressively when the extracellular pH is raised from 5.5 to 9.5. An increase of the intracellular pH from about 7.25 to about 7.95, calculated from external pH and the Donnan ratio, shifts the pH/flux relationship to the right, e.g. decreases the rate of efflux for a given extracellular pH. These findings can be rationalized in terms of a model (Fig. 11, model 1) in which the transport system exchanges lactate against OH^-. The stimulating effect of an extracellular alkalinisation would here result from the increasing concentration of an extracellular exchange partner for internal lactate. This would allow a carrier mediating a lactate/OH− exchange to return to the inside at a higher rate. The decrease of efflux with intracellular alkalinisation would result from the increase of the concentra-

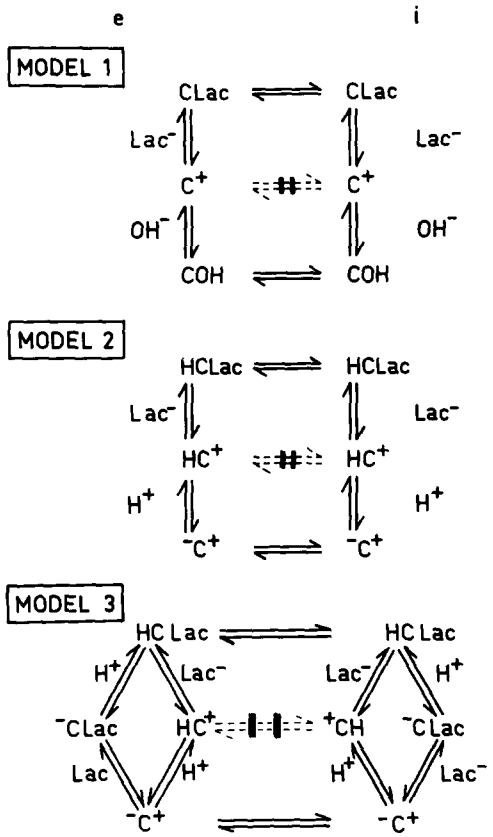

Fig. 11. Models of the mechanism of lactate movements across the human erythrocyte membrane. Model 1. A system, unable to 'move' when bearing a positive charge (C^+) but moving when complexed with lactate (Lac^-), or OH^-. Model 2, 3. A system able to 'move' in the unloaded ($^+C^-$) form or when loaded with H^+ and Lac^-, but unable to move when complexed only with H^+ or lactate (Lac^-).

tion of a competitor (OH^-) for lactate at the internal membrane surface. Alternatively, in terms of a lactate-H^+ cotransport model (Fig. 11, model 2 or 3), involving either an ordered or a random combination of the transport system with H^+ and lactate, extracellular alkalinisation would favour the formation of the deprotonated carrier, $^-C^+$, which is able to reorient in the membrane. This would again facilitate the return of the carrier to the inner surface. Intracellular alkalinisation would inhibit efflux due to a decreased availability of HC^+, the form of the carrier able to bind and translocate internal lactate.

As an alternative to these interpretations one might postulate that the pH dependency reflects changes of intrinsic properties of the transport system. This interpretation, however, is rather unlikely, since the pH-dependency of lactate equilibrium exchange differs from that of net flux [11] in a way predicted by the models in Fig. 11.

The different models also allow more quantitative predictions concerning the effects of separate and synchronous changes of pH on both sides of the membrane on the kinetic constants (K_T, V) of lactate transport, which provides a possibility to distinguish at least between models 2 and 3. Studies directed at this aim are presently under way.

In conclusion, the lactate transfer system and the inorganic-anion exchange system thus differ in one additional important respect: movements via the inorganic anion transfer system occur almost exclusively by a tightly coupled exchange of two anions. The lactate transport system does not require such a tight coupling, but mediates movements of lactic acid. This mechanism is very appropriate if one considers that both, lactate and protons, formed in parallel by glycolytic metabolism, can thus be removed in one single step.

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