Biochimica et Biophysica Acta, 507 (1978) 137—155

BBA 77912

STEREOSELECTIVE, SH-DEPENDENT TRANSFER OF LACTATE IN MAMMALIAN ERYTHROCYTES

B. DEUTICKE, I. RICKERT and E. BEYER

Abt. Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule, D-5100 Aachen (G.F.R.)

(Received June 8th, 1977)

Summary

The equilibrium exchange of lactate and glycolate was studied in human and other mammalian erythrocytes. Both anions penetrate by a process exhibiting Michaelis-Menten kinetics, slower than chloride but faster than sulfate. L-Lactate exchanges faster than D-lactate due to a different half saturation constant (L-lactate 66 mM, D-lactate 357 mM). Maximal transfer rates are identical for both isomers. The extent of stereoselectivity is species-dependent.

Lactate and glycolate transfer share basic characteristics with inorganic anion transfer (anion dependency, sensitivity to amphiphilic inhibitors (e.g. salicylate), to aminoreagents and pronase) but differ in others (pH dependency, low sensitivity to disulfonate inhibitors (4-acetamido-4′-isothiocyano stilbene-disulfonate, tetrathionate)). Activation energies are high (30—36 kcal/mol) at low temperature, but decrease (to 15—26 kcal) at higher temperature, breaks occurring between 10 and 20°C.

In marked contrast to inorganic anion transfer, lactate and glycolate transfer are inhibited reversibly by impermeable and permeable SH-reagents of the mercurial ($HgCl_2$, *p*-chlormercuribenzene sulfonate (PCMBS)) and the dithiol (4,4′-dithiodipyridine, 5,5′-dithiobis(2-nitrobenzoate)) type. The inhibition is incomplete, reversible and varies in its extent from anion to anion. PCMBS acts instantaneously, indicating a very superficial localization of the SH-group involved.

The results suggest that lactate and glycolate penetrate the erythocyte membrane predominantly, although not exclusively, via an anion transfer system specialized for this type of anion, and different from the classical inorganic anion exchange system.

Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzene sulfonic acid; PCMB, *p*-chloromercuribenzoate; PCMBS, *p*-chloromercuriphenylsulfonate; DTNB, 5,5′-dithiobis(2-nitro-benzoic acid); DTDP, 4,4′-dithiodipyridine; SITS, 4-acetamido-4′-isothiocyanostilbene disulfonate; SDS, sodium dodecyl sulfate.

Introduction

The mechanisms and pathways by which inorganic anions penetrate the erythrocyte membrane have aroused growing interest during the past decade. In numerous studies it could be demonstrated that rapidly penetrating monovalent anions (e.g. Cl^-) as well as slowly penetrating divalent anions (e.g. sulfate and phosphate) exhibit complex transfer kinetics, characterized by a high activation enthalpy, saturation kinetics, substrate inhibition, competitive inhibition by other anions and non-competitive inhibition by numerous chemically unrelated amphiphilic compounds [1—5].

Moreover, anion fluxes are inhibited by aminoreagents, e.g., fluorodinitrobenzene or pyridoxalphosphate [6,7]. Binding studies with these [8] and other covalent inhibitors, in particular stilbene disulfonates [9,10], support the idea that the major membrane-spanning protein of the erythrocyte membrane is involved in anion transfer. This 100 000-dalton fraction [12] comprises about 25% of the membrane proteins. Its homogeneity is still a matter of debate and the precise mechanism by which it mediates inorganic anion translocation far from clear.

In contrast to the advanced characterization of inorganic anion transfer, the transfer of monovalent organic anions has been studied to a much lesser extent [13—17] although some of them, e.g. lactate, pyruvate or the simple fatty acid anions, deserve considerable physiological interest. The characterization of the transfer of monovalent carboxylate anions is complicated by the fact that many of them penetrate biological membranes by nonionic diffusion of the undissociated acid. Whether this mechanism contributes to the transfer of a given anion in a particular membrane cannot be predicted from its chemical structure but has to be tested experimentally. A simple procedure, based on the measurement of proton movements and subsequent OH-Cl exchanges associated with the diffusion of undissociated acids into erythrocytes in unbuffered suspensions, was recently elaborated in our laboratory [13]. According to the results, later confirmed by Aubert and Motais [15], most unsubstituted, as well as α-hydroxy- and α-oxo-substituted aliphatic monocarboxylates penetrate the erythrocyte membrane to a considerable extent by nonionic diffusion. Exceptions are pyruvate, lactate, and its C_2-homologue, glycolate. The latter two anions were selected for an investigation of the ionic transfer of monovalent organic anions. It was the particular aim of this study to clarify whether these anions share the transfer system of the inorganic anions or have a special pathway for themselves. Preliminary results have been reported elsewhere [18,19].

Methods and Materials

The transfer of lactate and glycolate was studied by measuring tracer fluxes under equilibrium conditions in intact erythrocytes. For special purposes resealed ghosts were also used.

Tracer efflux measurements

Experiments were carried out with fresh human blood obtained at the local blood bank, with fresh blood from rat, rabbit, dog, cat and sheep, anticoagulated

with heparin, and with ox and pig blood obtained at the local slaughterhouse and anticoagulated with sodium citrate. After removal of plasma and buffy coat the erythrocytes were washed three times in 154 mM NaCl. In routine experiments the cells were then suspended at a hematocrit of 5% in one of the two following media (values in mM):

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

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

Incubation in medium A preserves the characteristic Donnan distribution of anions between cells and medium. In medium B cation impermeability is abolished by gramicidin B [20] and colloid-osmotic hemolysis prevented by extracellular sucrose [3]. The anion distribution ratio approaches unity. Cells were incubated in medium B whenever an increase of cation permeability was to be expected under the experimental conditions chosen.

Equilibration of the cells with these media, loading with [^{14}C]lactate, [^{14}C]-glycolate or [^{35}S]sulfate, measurements of isotope efflux into isotope-free but otherwise identical media and calculation of efflux rate coefficients followed essentially the protocol given in ref. 20. Fluxes, J, (mol $\cdot$ cm^{-2} $\cdot$ s^{-1}) were calculated from rate coefficients by the formula:

$$J = 3.5 \cdot 10^{-10} \cdot (DW^{-1} - 1) \cdot C_{ex} \cdot r_D \cdot k.$$

where DW = fractional dry weight of the cells at the end of the flux measurement, C_{ex} = extracellular concentration of the anion, r_D = Donnan ratio as calculated from the isotope distribution after establishment of tracer equilibrium and k = rate coefficient. This calculation procedure is basically that of Gardos et al. [21] but relates fluxes to unit area using a mean erythrocyte volume of 87 μm^3 at a fractional dry weight of 0.35 and a surface area of 142 μm^2/cell. Further details concerning the variation of incubation parameters and exposure to inhibitors are described in Results.

Pretreatment of cells with covalent modifiers

Cells were suspended at a hematocrit of 20% in the following medium (values in mM): KCl, 90; NaCl, 45; Na_2HPO_4/NaH_2PO_4, 12.5; sucrose, 40. After pH adjustment the modifiers were added in a small volume of solvent. Following the appropriate period of exposure the cells were washed three times with the above medium and immediately used for flux measurements.

Tracer influx measurements

Freshly obtained human erythrocytes were suspended at a hematocrit of 7% in media containing (concentrations, mM): KCl, 100; NaCl, 35; Na_2HPO_4/NaH_2PO_4, 6.5; glucose, 2.5; lactate, 6.5, and equilibrated for 60 min at pH 7.2 and 37°C. The suspensions were then centrifuged and the supernatants removed by aspiration to obtain a hematocrit of 40%. The suspension was cooled to 10°C and [^{14}C]lactate added. 4-ml samples were pipetted at 1-min intervals into 46 ml ice-cold 'stop solution' of the following composition (mM): KCl, 100; NaCl, 42; Na_2HPO_4/NaH_2PO_4, 6.5; glucose, 2.5; phloretin, 0.25 mM or salicylate, 20 mM. In such solutions the fractional loss of radioactivity amounts

to less than 0.0001 min^{-1} according to preliminary studies. The cells were centrifuged, washed three times at 0°C in the stop solution and extracted with a 3-fold excess of 0.6 N $HClO_4$. 1.0 ml of protein-free supernatant as well as 0.1 ml of the influx medium, deproteinized by addition of 0.001 ml of 60% $HClO_4$, were used for the determination of radioactivity by liquid scintillation counting. The uptake of radioactivity (per unit volume of cell water) was expressed as percent of the extracellular radioactivity. This uptake proved to increase almost linearly with time during the initial period studied and could therefore be used as a relative measure of lactate equilibrium exchange under the standardized conditions.

Materials

Routine chemicals were of the highest purity available. Lactate was obtained as sodium L-lactate (Fluka 71718, purum) and lithium L- or D-lactate (Calbiochem 427052, 'A grade'), used without any further purification. Glycolic acid was from Fluka (50590, puriss.) L[U-^{14}C]Lactate and D[U-^{14}C]-lactate, [1-^{14}C]glycolate and [^{35}S]sulfate (sodium salts) were obtained from Amersham Buchler. According to the manufacturer's control sheets the radiochemical purity is better than 98%.

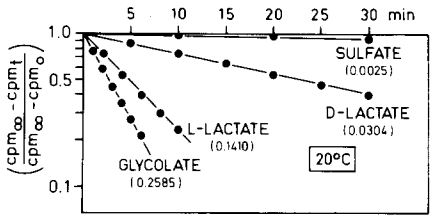
1-Fluoro-2,4-dinitrobenzene, sodium pyridoxalphosphate, *p*-chloromercuribenzoic acid sodium salt, 5,5′-dithiobis(2-nitrobenzoic acid), pronase (70 000 PUK */g), reduced glutathione and L-cysteine were obtained from Merck, Darmstadt. *p*-Chloromercuriphenylsulfonic acid, sodium salt and 2,4,6-trinitrobenzene sulfonic acid were from Sigma, St. Louis. 4,4′-dithiodipyridine and 2-mercaptoethanol were from Fluka AG, Buchs. Dithioerythritol and 4-acetamido-4′-isocyanostilbene disulfonic acid, sodium salt, were from Serva, Heidelberg, and phloretin from Roth, Karlsruhe. Gramicidin B was kindly donated by von Heyden-Squibb Munich, and phenyopyrazone (1,4-diphenyl-3,5-dioxopyrazolidine) was a gift from Knoll AG, Ludwigshafen.

Results

Stereoselectivity of lactate transfer

In order to relate the permeation rates of glycolate and lactate to those of inorganic anions, the tracer fluxes of these organic anions and of sulfate were measured under identical conditions. The rate coefficient of glycolate efflux was two orders of magnitude higher than that of sulfate (Fig. 1). In case of lactate a considerable difference between the L- and the D-isomer became evident. Tracer efflux from L-[^{14}C]lactate-loaded erythrocytes was about five times faster than tracer efflux from D-[^{14}C]lactate loaded cells. Both fluxes were slower than those of glycolate but faster than those of sulfate. This difference between the two isomers suggests stereoselectivity of the lactate transfer system. However, the observed effect could also be due to artifacts, arising from a preferential efflux of [^{14}C]pyruvate formed, by isotope exchange via lactate dehydrogenase, from L-[^{14}C]lactate but not D-lactate during the loading period [22]. Since pyruvate efflux is much faster than D-lactate efflux

* PUK, proteolytic units according to M. Kunitz [69].



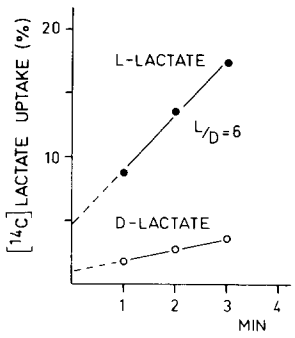


Fig. 1. Time course of the equilibrium exchange of anions, as measured by tracer efflux from preloaded human erythrocytes. Cells equilibrated with the various anions in Medium A (see Methods), pH 7.4. Efflux initiated by mixing tracer loaded-cells with tracer-free medium of identical composition. Ordinate: Extracellular radioactivity (per 0.5 ml medium) after various intervals (cpm_t) in relation to extracellular radioactivity at time zero (cpm_0) and after attainment of tracer equilibrium (cpm_∞). Rate coefficients (min^{-1}), calculated under the assumption of an apparent first order kinetics, in parentheses.

Fig. 2. Stereoselectivity of [^{14}C]lactate uptake, at Donnan equilibrium, into human erythrocytes. 10°C, pH 7.35, extracellular lactate 6.5 mM, hematocrit 40%. For further details see Methods.

(Deuticke, unpublished results), such an isotope exchange might produce falsely high transfer rates of L-lactate. Three types of experiment were carried out to exclude this artifact.

1. Experiments on resealed ghosts. Erythrocyte ghosts prepared, with minor modifications, according to Lepke and Passow [23] were equilibrated with D- and L-lactate and tracer efflux was measured as described for intact cells. In spite of the fact that in these resealed ghosts the content of enzyme and coenzyme required for conversion of L-[^{14}C]lactate into pyruvate is lowered by a factor of 20, tracer efflux was 4.5 times faster in L-lactate-loaded than in D-lactate-loaded cells ($k_{\text{L-lactate}} = 0.1238$; $k_{\text{D-lactate}} = 0.0267$ min^{-1}, 20°C).

2. Dinitrophenylhydrazine precipitation. If [^{14}C]pyruvate were released from L-[^{14}C]lactate-loaded cells, it should be possible to precipitate radioactive material in the external medium by a carbonyl-specific reagent such as dinitrophenylhydrazine. Following the procedure of Silverstein and Boyer [24], however, radioactive dinitrophenylhydrazones could not be detected in the efflux media.

3. Tracer influx experiments. The influx technique described in the Method precludes conversion of L-[^{14}C]lactate into [^{14}C]pyruvate prior to membrane passage. As shown in Fig. 2, under these conditions, too, L-lactate penetrates the erythrocyte membrane much faster than D-lactate.

It therefore seems safe to postulate a stereoselective lactate transfer system in the erythrocyte membrane. This stereoselectivity was also observed in other mammalian species (Table I). It is most pronounced in rat and rabbit erythrocytes, but lacking in ox and sheep erythrocytes. Table I also demonstrates marked species differences in the rates of lactate transfer. These differences are even larger than indicated by the data given, since fluxes had to be measured at different temperatures for technical reasons. After correction on the basis of the temperature dependency of lactate transfer, L-lactate turns out to penetrate about 2000-times faster in rat erythrocytes than in sheep

TABLE I

STEREOSELECTIVITY OF LACTATE EQUILIBRIUM EXCHANGE IN VARIOUS MAMMALIAN ERYTHROCYTES

Fluxes measured in Medium A (see Methods), pH 7.35, lactate concentration 5.4 mM. Mean values (min^{-1}) ± S.D. from 3—6 experiments.

	Rate of equilibrium exchange of:		
	L-Lactate	D-Lactate	Ratio L/D
Rat (0°C)	0.4622 ±0.1081	0.0212 ±0.0062	21.8
Rabbit (0°C)	0.4915 ±0.0376	0.0291 ±0.0086	16.9
Dog (10°C)	0.2345 ±0.0274	0.0378 ±0.0055	6.2
Man (20°C)	0.1487 ±0.0193	0.0296 ±0.0040	5.0
Cat (20°C)	0.0919 ±0.0142	0.0147 ±0.0040	6.3
Pig (25°C)	0.0470 ±0.0069	0.0187 ±0.0021	2.5
Ox (25°C)	0.0110 ±0.0032	0.0100 ±0.0025	1.1
Sheep (25°C)	0.0068 ±0.0003	0.0069 ±0.0008	1.0

erythrocytes. The same ratio amounts to 10 for phosphate [25] and to only 4 for chloride [26].

Basic characteristics of lactate and glycolate equilibrium exchange

In order to characterize the transfer system of lactate and glycolate, a number of parameters known to affect the pathway for inorganic anions were studied. The presence or absence of similarities between organic and inorganic anions was expected to provide some indication concerning the question of a common pathway.

Temperature. The transfer of divalent anions exhibits a remarkably high temperature dependency. Q_{10} values between 5 and 7, corresponding to apparent activation energies (E_a) of 30—36 kcal/mol, have been reported [1,2]. The same is true for chloride transfer at low temperatures [4], while at higher temperatures the activation energy drops to 22 kcal/mol, due to a discontinuity in the region of 15°C [27]. As is evident from the Arrhenius plots in Fig. 3, the equilibrium exchange of lactate and glycolate behaves in a similar way. At low temperatures the rate coefficients increase steeply and at higher temperatures only moderately with increasing temperature. Breaks occur between 10° and 20°C. This finding correlates with the observation of a discontinuity of the activation energy of pyruvate transfer at 10°C [16].

Concentration dependency. Equilibrium fluxes of sulfate and chloride exhibit saturation kinetics superimposed by substrate inhibition [3,4]. Apparent half-saturation constant (K_T) values of approx. 20—40 mM have been

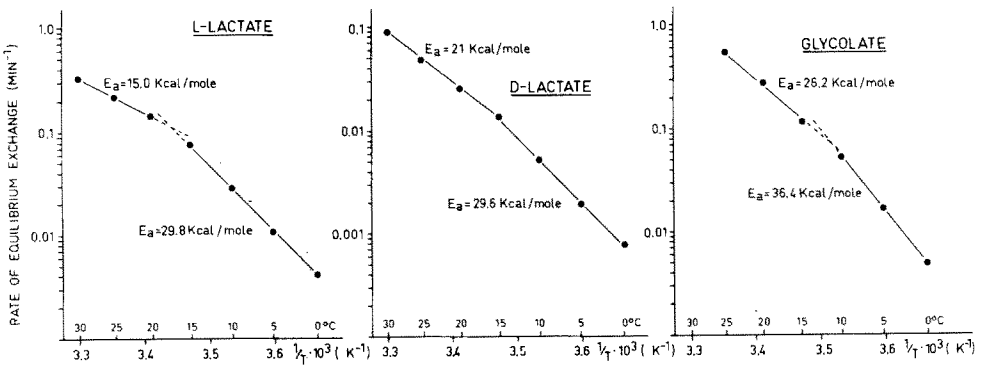


Fig. 3. Arrhenius diagrams for the equilibrium exchange of lactate and glycolate. Fluxes measured in Medium A, pH 7.35. Apparent activation energies derived from the slope of linear regressions calculated from mean values of three experiments for each anion.

reported for both anions in spite of large differences in the maximal transfer rates (J_{max}). The fluxes of glycolate and lactate also saturate in a fashion describable by Michaelis-Menten kinetics *. K_T values, however, derived from Lineweaver-Burke ($1/J$ vs. $1/c$) diagrams (Fig. 4) and substantiated by other linearization procedures (J/c vs. J, c/J vs. c) are higher than those for sulfate and chloride. This is particularly true for D-lactate: a half-saturation constant of 357 mM indicates a very low affinity. J_{max} values for L- and D-lactate (7.1 pmol · cm^{-2} · s^{-1} at 20°C) are identical. Consequently, the differing rates of L- and D-lactate transfer are most likely due to different affinities of both anions for a common transfer system. The ratio of the K_T values, 357 : 66 (= 5.4), therefore defines more precisely than the flux ratio given above the stereoselectivity of lactate transfer. J_{max} for glycolate is ten times higher than J_{max} for the lactates. No evidence for substrate inhibition was obtained in the concentration range of the present study.

pH-Dependency. The pH-dependencies of inorganic transfer have been studied extensively [1—5]. Divalent anions exhibit a pronounced maximum at pH 6.4. In contrast, chloride fluxes increase with pH between pH 5 and 7 but remain constant at higher pH values [28]. The pH dependencies of glycolate and lactate transfer do not agree with either of these pH dependencies and even differ from each other (Fig. 5). Glycolate fluxes reach a flat maximum between pH 5.8 and 7.8; fluxes of D- and L-lactate increase progressively with lowering of pH. These pH dependencies cannot be explained straightforwardly by any of the current models of anion transfer. It cannot be excluded that nonionic diffusion via the lipid phase of the membrane contributes to the increase of lactate fluxes at low pH (see also ref. 29). The lipid phase might be less accessible to glycolate, in spite of the identical pK values of both acids, due to differences in lipid solubility (K_{ether}: lactate 0.09; glycolate 0.03 [30]).

Influence of the anion milieu. Anions interfere considerably with the

* Fluxes were measured in anisoosmotic media in order to avoid interference by other anions. The presence of gramicidin, which makes the cells leaky for cations, prevented osmotic responses of the cells.

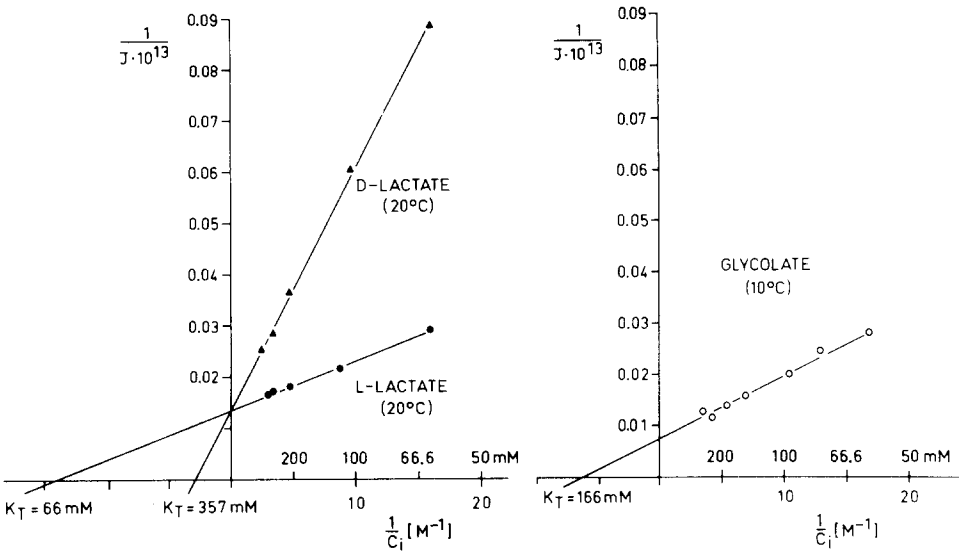


Fig. 4. Lineweaver-Burke plots for the concentration dependency of lacate and glycolate equilibrium exchange in human erythrocytes. Fluxes J (mol · cm^{-2} · s^{-1}) measured in media containing, besides the anion studied (concentrations, mM): Na_2HPO_4/NaH_2PO_4, 6.4; sucrose, 44; gramicidin, 5 μg/ml (see also ref. 3). Intracellular anion concentrations (C_i) were calculated from the isotope distribution ratio after attainment of equilibrium and the extracellular anion concentration. K_T (concentration at half maximal flux) values derived from linear regressions. Mean values from three experiments for each anion.

transfer of inorganic anions as indicated, e.g., by marked changes of transfer rates after replacement of chloride in the medium by other anions [2—4]. The same is in principle true for lactate and glycolate (Table II) although the pattern of anion effects differs from that of sulfate transfer. While replacement of chloride by nitrate produces the same effect on the transfer of all the anions (approx. 50—75% inhibition) different effects are observed with other anions.

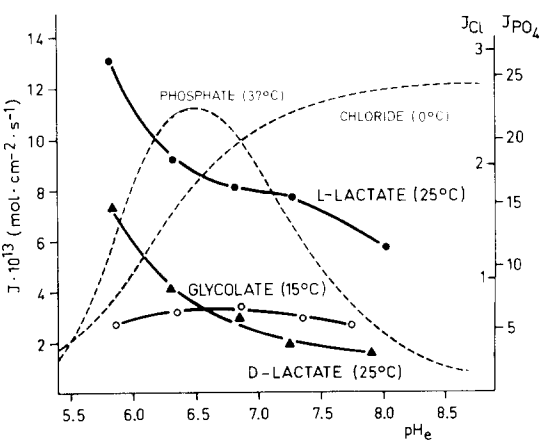


Fig. 5. pH Profile of the equilibrium exchange of lactate and glycolate in human erythrocytes. Fluxes measured in Medium B, (see Methods); lactate or glycolate concentration, 5 mM. pH Profiles of phosphate (2) and chloride (25) equilibrium exchange are shown for comparison. J_{PO_4}: mol · g cells^{-1} · min^{-1} × 10^9; J_{Cl}: mol · g cells^{-1} · min^{-1} × 10^3.

TABLE II

INFLUENCE OF THE ANION MILIEU ON THE EQUILIBRIUM EXCHANGE OF INORGANIC AND ORGANIC ANIONS

Human erythrocytes were loaded (hematocrit 5%) with labelled sulfate (3.5 mM), glycolate or lactate (5 mM) in Medium A containing chloride, nitrate, acetate and propionate (all 140 mM) respectively, or malonate (95 mM) as the major anion. Efflux measured at a hemotocrit of 5%, into identical, but tracer-free media.

Main anion of incubation medium	Relative rate of equilibrium exchange of:			
	Sulfate (35°C)	Glycolate (15°C)	D-Lactate (20°C)	L-Lactate (20°C)
Chloride	1.00	1.00	1.00	1.00
Nitrate	0.32	0.60	0.56	0.50
Malonate	3.80	2.14	1.80	1.64
Acetate	1.61	1.16	0.64	0.68
Propionate	1.62	0.91	0.30	0.20

Replacement of chloride by malonate, acetate or propionate considerably enhances sulfate transfer, indicating that these organic anions have a lower affinity than chloride for the binding sites involved in sulfate transfer. In contrast, glycolate and lactate fluxes are much less enhanced or even inhibited after replacement of chloride by the organic anions.

Influence of non-covalent and covalent inhibitors. Salicylate, tetracaine, phloretin and phenopyrazone have previously been shown to be potent reversible inhibitors of the transfer of Cl^- and divalent inorganic anions [2,3,32]. As is evident from Fig. 6 and Table III, these amphiphilic compounds

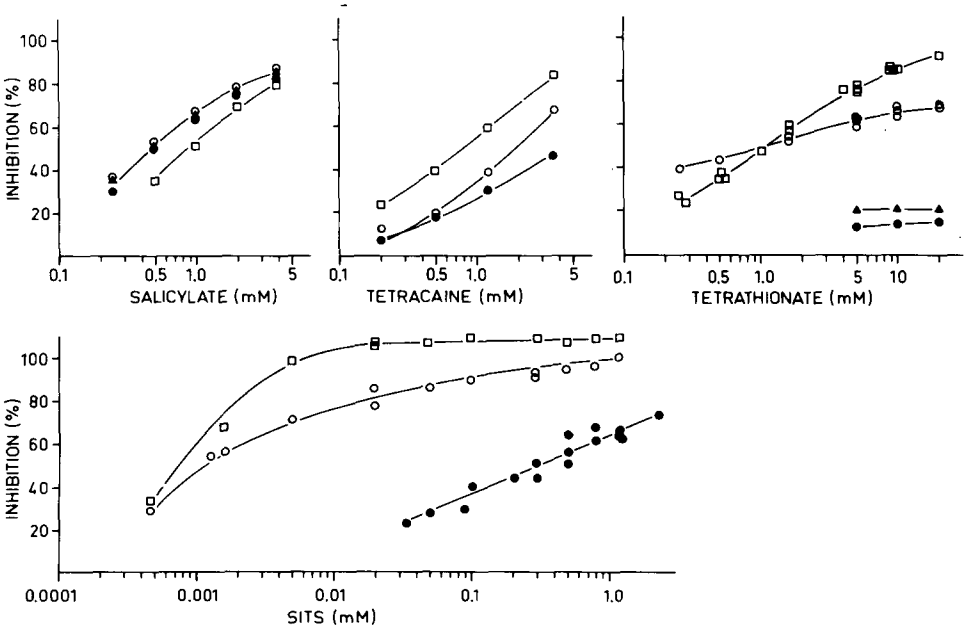


Fig. 6. Influence of inhibitors of inorganic anion transfer on the equilibrium exchange of lactate and glycolate. Inhibitors were present only during tracer efflux measurement. Anion concentration 5.4 mM, Medium A, pH 7.4. □, Sulfate (35°C); ○, glycolate (15°C); ▲, D-lactate (20°C); ●, L-lactate (20°C).

TABLE III

INFLUENCE OF MEMBRANE MODIFIERS AND AMPHIBILIC INHIBITORS ON THE TRANSFER OF VARIOUS ANIONS ACROSS THE HUMAN ERYTHROCYTE MEMBRANE

Inhibitor (mM)	Percent inhibition of equilibrium exchange of:		
	Sulfate (35°C)	Glycolate (15°C)	L-Lactate (20°C)
FDNB (3.5) *	92	83	95
TNBS (1.0) *	59	68	36
Pronase (2.5 mg/ml) *	86	67	47
Pyridoxalphosphate **	86	40	51
Phenopyrazone (7.5) **	99	94	90
Phloretin (0.6) **	90	91	94

* Cells pretreated for 60 min, pH 7.4, 37°C.
** Inhibitor present during efflux period.

also inhibit the fluxes of lactate and glycolate. Furthermore, exposure to aminoreactive agents such as pyridoxalphosphate [7] FDNB [6] or TNBS, as well as extensive treatment with pronase, known to inhibit inorganic anion transfer [33], produces a marked inhibition of lactate and glycolate transfer. It should be noted, however, that TNBS and pronase loose inhibitory potency in the order sulfate > glycolate > lactate. This is even more true for SITS and tetrathionate, two potent inhibitors of sulfate and chloride transfer [34,35], which are only weak inhibitors of lactate transfer (Fig. 6).

The inhibitory effect of SH-reagents

SH-reagents do not affect the fluxes of sulfate [36] and chloride (Deuticke, unpublished results) in human erythrocytes. In contrast, the transfer of lactate and glycolate is markedly inhibited (Table IV) by $HgCl_2$, organomercurials (PCMBS or PCMB) or dithiols (DTNB or DTDP). Alkylating SH-reagents (iodoacetate, iodoacetamide) or disulfide forming agents (diamide, tetrathionate), on the other hand, have only minor effect (data not shown).

The inhibitions shown in Table IV are the maximal ones obtainable after pretreatment with the agents, as shown in detail for PCMBS in Fig. 7. Obviously, L-lactate is most sensitive (maximal inhibition 90%) followed by D-lactate (60%) and glycolate (25%). The rates of the PCMBS-insensitive residual transfer of L- and D-lactate are almost equal (Table IV).

Sensitivity to PCMBS is also observed in other mammalian species (Table V). The species sequence of PCMBS sensitivity parallels that of the stereoselectivity which indicates that both phenomena are causally related.

Characteristics of the inhibitor effects of SH-reagents

Reversibility. Organic mercurials and dithiols combine with SH-group reversibly [37]. Normalization of erythrocyte membrane permeabilities after removal of these agents has been demonstrated [38,39]. Inhibition of lactate transfer by PCMBS could be partly relieved by exposing PCMBS- and DTNB-treated cells to various thiols (Table VI). The antagonists varied in their restitutive potency, inhibition by DTNB was counteracted only by dithioery-

TABLE IV

INFLUENCE OF SH-REAGENTS ON THE EQUILIBRIUM EXCHANGE OF ANIONS

Fluxes measured in Medium B (see Methods). Cells pretreated with the organic reagents as described in the Methods; $HgCl_2$ present only during efflux.

Inhibitor (μmol/ml cells)	Rate of equilibrium exchange (min^{-1}) of:							
	L-Lactate (25°C)	Percent of control	D-Lactate (25°C)	Percent of control	Glycolate (15°C)	Percent of control	Sulfate (35°C)	Percent of control
None	0.1640		0.0399		0.0615		0.0140	
$HgCl_2$ (3.0), pH 7.4	0.0321	19	0.0165	41	0.0840	78	0.0125	90
P-Chloromercuriphenylsulfonate (3.0), pH 7.4	0.0203	12	0.0171	43	0.0440	72	0.0140	100
5,5′-Dithiobis(2-nitrobenzoate) (30), pH 8.0	0.0251	15	0.0160	40	0.0395	64	0.0140	100
4,4′-Dithiodipyridine (30), pH 8.0	0.0210	13	—	—	0.0360	58	0.0130	93

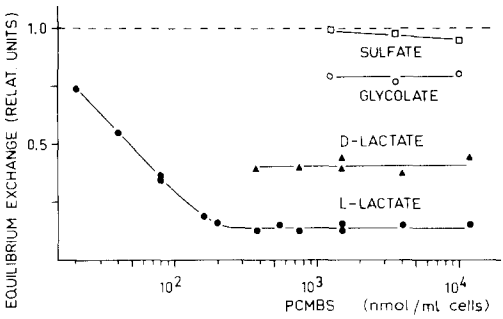


Fig. 7. Different extent of inhibition of anion equilibrium exchange after pretreatment of human erythrocytes with PCMBS. Cells (Hct 20%), pretreated for 90 min, pH 7.4, 37°C). Further details as in Table IV.

TABLE V

STEREOISOMER SELECTIVITY AND PCMBS SENSITIVITY OF LACTATE TRANSFER IN VARIOUS SPECIES

	$\frac{k\text{L-Lactate}}{k\text{D-Lactate}}$	Maximal inhibition of L-lactate transfer by PCMBS (%)
Rat (0°C)	21.8	95
Rabbit (0°C)	16.8	95
Dog (10°C)	6.2	70
Man (20°C)	5.0	90
Cat (20°C)	6.3	80
Pig (25°C)	2.5	50
Ox (25°C)	1.1	15
Sheep (25°C)	1.0	0

TABLE VI

REVERSIBILITY OF THE INHIBITION OF LACTATE TRANSFER BY SH-REAGENTS

Cells were first treated with the inhibitors as described in the Methods, and then loaded with [^{14}C]lactate in presence of the various thiols. Thiol concentrations 100 μmol/ml cells.

	Rate coefficients of equilibrium exchange (min^{-1})	
	L-Lactate (20°C)	D-Lactate (20°C)
Control	0.1610	0.0398
PCMBS 3.0 μmol/ml cells *	0.0210	0.0150
PCMBS then cysteine **	0.0620	0.0300
PCMBS then mercaptoethanol **	0.1410	0.0300
PCMBS then dithioerythritol **	0.1140	0.0270
Control	0.1373	
DTNB 25 μmol/ml cells ***	0.0342	
DTNB then cysteine **	0.0339	
DTNB then mercaptoethanol **	0.0321	
DTNB then dithioerythritol **	0.0720	

* 30 min, pH 7.4, 37°C.
** 150 min, pH 7.4, 37°C.
*** 150 min, pH 8.5, 37°C.

thritol. In addition, the effects of DTNB could be partly relieved by sulfitolysis of the S-S bonds.

Time course. PCMBS and DTNB penetrate the diffusion barrier of the erythrocyte membrane only very slowly [40,41]. For this reason the inhibition of lactate transfer should develop with delay if the SH-groups involved in the transfer were located behind that barrier. As shown in Fig. 8a, the half-time for inhibition of lactate transfer by DTNB amounts to 20 min, maximal inhibition requires more than 120 min. PCMBS, however, exerts its full effect within less than 5 min. This could be established more precisely by two types of experiment. In the first one (Fig. 8b) PCMBS was added to the erythrocyte suspension during the tracer efflux period. The intercept of the regression for the altered efflux kinetics with the regression prior to the addition of inhibitor coincided with the point at which PCMBS was added. In the second type of experiment (Fig. 9), [^{14}C]lactate efflux was started in the presence of an amount of PCMBS just sufficient to produce maximal inhibition. Small excess amounts of glutathione were then added to parallel samples after various brief intervals in order to remove PCMBS not yet bound to the erythrocytes. Inhibition by PCMBS reached 50% within less than 2 s in spite of the considerable mixing problems of the system.

Considering the low permeability of PCMBS, the SH-groups involved in lactate transfer may therefore be assumed to be located at the outer surface of the erythrocyte membrane. The slow inhibition by DTNB is probably due to its sluggish reaction with these SH-groups, since DTDP, a readily permeable analogue of DTNB [41], also inhibits lactate transfer more slowly than PCMBS (see Fig. 8a).

In view of the rapid interaction of PCMBS with its superficial membrane binding sites it was possible to study the dose response curves of this and other mercurials in cells only exposed to the inhibitor during the efflux period. This type of experiment allows a precise relation of the extent of inhibition to extracellular inhibitor concentrations.

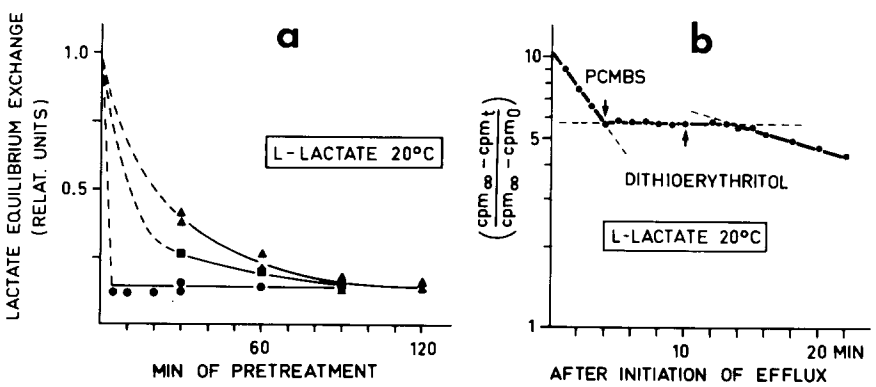


Fig. 8a. Time course of the development of the inhibition of lactate equilibrium exchange during pretreatment with ●, PCMBS (3.0 μmol/ml cells, pH 7.4); ■, DTDP and ▲, DTNB (30 μmol/ml cells, pH 8). Fluxes measured in Medium B. b. Time course of the inhibition of lactate equilibrium exchange by PCMBS (12 μmol/ml cells) added during the efflux measurement period, and of its partial relief by addition of dithioerythritol (400 μmol/ml cells). Fluxes measured in Medium B, pH 7.4 (Hct 5%).

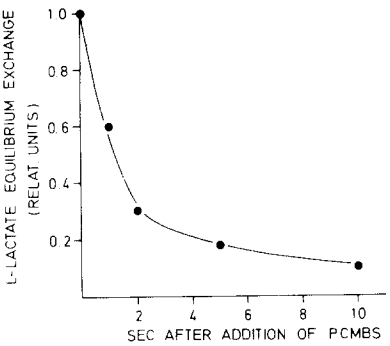


Fig. 9. Time resolution of the development of inhibition of lactate transfer by PCMBS (0.05 μmol/ml cells) added 2 min after initiation of efflux (20°C, pH 7.4). Glutathione (0.2 μmol/ml cells) was added either together with, or 1, 2, 5, 10 s after the PCMBS. The efflux was then followed for another 10 min. The efflux kinetics, obtained as in Fig. 8, were linear during this period, indicating the instantaneous removal of unbound PCMBS by the glutathione.

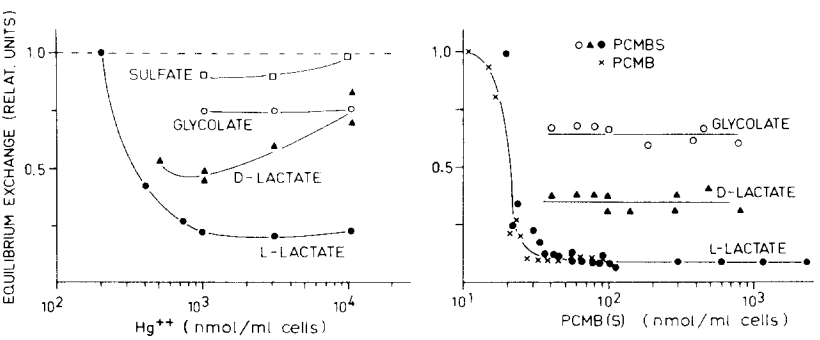


Fig. 10. Dose vs. response curve for the inhibition of anion transfer in the presence of mercurials. Fluxes measured in Medium B, pH 7.4, 20°C.

The blockage of lactate transfer in presence of PCMBS and PCMB (Fig. 10) is characterized by almost the same maxima observed in pretreated cells. The coincidence indicates that the incomplete inhibition is not due to partial elution of inhibitor by subsequent washing procedures. Inhibition is maximal at extracellular concentrations as low as 30—40 nmol/ml cells. Hg^{2+} ions produce an additional acceleration of the transfer at higher concentrations most evident for D-lactate. Such biphasic effects of Hg^{2+} have also been described for the facilitated diffusion of erythritol via the glucose transfer system [42].

Discussion

The characteristics of lactate and glycolate equilibrium exchange presented above indicate that a single anion transfer system in the erythrocyte membrane cannot a priori be taken for granted. On the other hand, a number of common properties of the transfer of inorganic and these organic anions argue against completely unrelated transfer mechanisms. This ambiguity has also become evident, while our work was in progress, in an analysis of pyruvate transfer in

resealed ghosts [17] and of pyruvate and lactate uptake in human erythrocytes [16]. These studies, however, were carried out under experimental conditions deviating from those of most studies on inorganic anion transfer. The present results allow a direct comparison. In the following, they will be analysed particularly with respect to the relationship between the transfer systems of inorganic anions and of lactate and glycolate.

The basic characteristics of lactate and glycolate transfer

The most conspicuous common transfer characteristic of the two groups of anions would seem to be their sensitivity to numerous inhibitors (Fig. 6, Table III). This similarity, already observed by Halestrap [16], also pertains to the transfer of pyruvate, which probably shares the pathway of lactate [16,17]. On the other hand, inhibitors like phloretin or FDNB also affect quite different transfer processes, e.g. of hexoses [44]; anionic inhibitors such as salicylate could also interfere with anion binding sites different from those involved in inorganic anion transfer and FDNB and pyridoxalphosphate bind to membrane proteins other than the 100 000-dalton fraction involved in inorganic anion transfer [8,45]. Moreover, specific disulfonate inhibitors of inorganic anion transfer, e.g. SITS or tetrathionate [34,35] inhibit lactate (see Fig. 6) and pyruvate [16] transfer only little. Therefore, the inhibitor data do not provide clear evidence for or against a common anion transfer system. The same is true for the different effects of the anion milieu, since different anion affinities of a common anion transfer system would be difficult to distinguish from patterns of anion competition observable with two different systems.

The temperature dependencies of lactate and glycolate transfer, likewise, do not provide a means for the discrimination of transfer pathways. The origin of the discontinuities in the Arrhenius plots, also reported for hexose equilibrium exchange in erythrocytes [46] (but see ref. 47), remains to be elucidated. For transport processes and enzyme activities in other membranes such breaks have been correlated with thermotropic phase transitions of membrane lipids [48—51]. In the erythrocyte membrane 'macroscopic' phase transitions (detectable by, for example, calorimetry) are suppressed by cholesterol [52]. However, 'microscopic' transitions taken to involve lipids directly apposed to intrinsic membrane proteins, have been reported [53—55]. The significance of such lipid-protein interactions for lactate transfer is also indicated by its sensitivity to membrane lipid perturbations by changes of the cholesterol content [5] or by enzymatic phospholipid cleavage [56].

The kinetic constants of lactate transfer deserve some comment: The K_T value for L-lactate in our study (66 mM) is considerably higher than that reported by Halestrap (0.9 mM) [16]. While different experimental conditions may contribute to these differences, it must also be considered that our K_T value was derived from flux measurements at high lactate concentrations, while the value given by Halestrap applies to low lactate concentrations. From a Lineweaver-Burk plot of his data Halestrap also inferred a much higher K_T value for lactate concentrations >20 mM. This finding was rationalized in terms of two parallel pathways for lactate, a 'low affinity system' identical with the chloride transfer system and a 'high affinity system' representing a more specific lactate/pyruvate carrier. A comparison of J_{max} values characterizes the

relative transport capacity of the human erythrocyte for lactate. Its J_{max} value at 25°C (10 μmol · g^{-1} · min^{-1}, or 10^7 ions/cell per s) is 1000 times lower than that of chloride (10^4 μmol · g^{-1} · min^{-1}, or 10^{10} ions/cell per s (computed from ref. 27)).

The basis of the species differences in lactate and glycolate transfer

The species sequence of erythrocyte permeabilities to nonelectrolytes penetrating via the membrane lipid phase is correlated with the mean number of double bonds of the membrane phospholipid fatty acids and with the membrane phosphatidylcholine content of the respective species [5,14]. This correlation has been interpreted by effects of the microviscosity or the interfacial properties of the lipid bilayer on transmembrane diffusion. Interestingly, the rates of phosphate [25] and chloride [26] exchange follow almost the same species sequence as nonelectrolyte transfer [5], which may indicate an influence of membrane lipids on anion transfer.

Qualitatively, the equilibrium exchange of lactate and glycolate exhibits the same species sequence as phosphate. The correlation between the fluxes of phosphate and these anions, however, is not linear (Fig. 11). A much steeper slope is observed for species with a rapid, stereoselective and PCMBS-sensitive lactate exchange (dog, rat, man) than for species with a slow, unselective and PCMBS-insensitive lactate exchange (sheep, ox, pig). Provided that the species sequences are related to effects of the membrane lipids, the lactate transfer system in the former group of erythrocytes would seem to be much more sensitive to its lipid environment than that in the latter group, supporting the presence of a specific pathway in the former group.

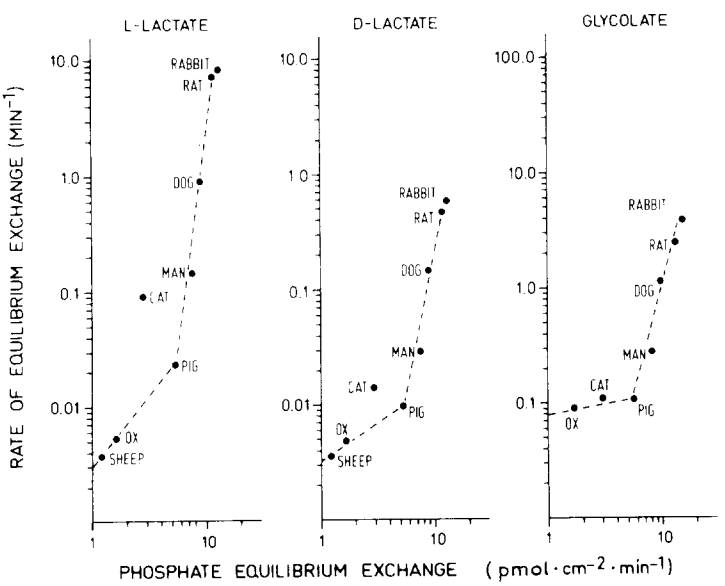


Fig. 11. Nonlinear correlation between the species sequences for the equilibrium exchange of phosphate [68] and lactate or glycolate. Rate coefficients for lactate and glycolate calculated for a temperature of 25°C from the data in Fig. 3 using mean activation energies of 33 (glycolate) 25 (D-lactate) and 23 (L-lactate) kcal/mol.

The effects of SH-reagents

In contrast to the characteristics discussed so far, inhibition of lactate and glycolate transfer by SH-reagents is a unique property of these, and probably similar, anions. The sensitivity of lactate transfer to SH-reagents and its extent of stereoselectivity parallel each other, (Table V). This phenomenon, taken together with the observation that:

1, inhibition by SH-reagents is incomplete;

2, the residual transfer rates after maximal inhibition are almost equal for both lactate isomers;

3, tetrathionate and stilbene disulfonates completely inhibit inorganic anion transfer, but have only minor effects on lactate transfer,

suggests two pathways of lactate transfer. One of these, stereoselective and SH-dependent, might be specialized on lactate and similar anions. The other one, SH-independent, unselective but highly sensitive to disulfonate inhibitors, could correspond to the 'classical' inorganic anion exchange system. While most of our data could be incorporated into such a model, the simple saturation kinetics of lactate and glycolate transfer remain difficult to reconcile with this concept.

Lactate transfer is inhibited instantaneously by the highly impermeable [40,41] PCMBS. This observation facilitates the localization of the SH-groups involved. According to Sutherland et al. [38], only approx. 3% of the erythrocyte membrane SH-groups, $8.5 \cdot 10^5$/cell, interact rapidly with PCMBS and may be assumed to reside at the exofacial membrane surface. The same number of SH-groups reacts with DTNB [57] and with the fully impermeable macromolecular SH-reagent, dextranmaleimide [58]. $8.5 \cdot 10^5$ sites/cell are probably an overestimate of the exofacial SH-groups involved in lactate exchange, since other transfer processes, e.g. of water, small nonelectrolytes and hexoses [39,59,60] also require such groups. The exofacial SH-groups involved in these other processes differ functionally from those required for lactate exchange: water and glycerol transfer are inhibited by PCMBS only slowly [39], while hexose transfer is insensitive to DTNB [41]. Only a subpopulation of the exofacial SH-groups may therefore participate in lactate transfer.

The protein bearing these SH-groups remains to be indentified. An obvious candidate might be the 100 000-dalton fraction assumed to mediate inorganic anion transfer. This fraction contains cysteine residues [61] and reacts with DTNB in the intact cell [62]. Two models could reconcile the different responses of lactate and inorganic anion transfer to SH-reagents and their common sensitivity to aminoreagents with a joint transfer via the 100 000-dalton fraction. On the one hand, this fraction, which seems to be heterogeneous in composition [63], might contain one protein mediating the transfer of chloride, sulfate (and also lactate), and another one specific for lactate and similar anions. Alternatively, the transfer of all these anions might occur via a single protein with an NH_2-containing region required by all anions and an additional domain bearing an SH-group involved in lactate transfer but dispensable for the transfer of the inorganic anions.

In contrast to such unifying models, the SH-dependent stereoselective transfer might also be catalysed by an unrelated protein. According to SDS

gel electrophoretic analyses of dextranmaleimide-treated erythrocytes [58], exofacial SH-groups are localized predominantly in the region of proteins with apparent molecular weights of 50 000—60 000 and 30 000 Daltons (bands 4.5 and 7 according to Steck [11]). In preliminary binding studies with [^{14}C]PCMB we have observed a corresponding labelling pattern. Such data support the concept of a second anion transfer system in the erythrocyte membrane additional to that mediating the transfer of inorganic anions. Similar systems may be operative in other cell membranes as indicated by data obtained on intestinal [64], ascites tumor cells [65], cardiac [66] and sceletal [67] muscle, and the blood-brain barrier [68].

Acknowledgement

Supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 160).

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