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THE ROLE OF CARBONIC ANHYDRASE INHIBITORS ON ANION PERMEABILITY INTO OX RED BLOOD CELLS

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SUMMARY

1. Organic anion permeability in ox red blood cell was measured by studying steady-state self-exchange of oxalate, chosen as a prototypical substrate of the organic anion transport system previously described; chloride self-exchange measured the inorganic anion permeability.

2. Carbonic anhydrase inhibitors of the sulphonamide class inhibit both organic anion self-exchange (A^-/A^-) and chloride self-exchange (Cl^-/Cl^-) although carbonic anhydrase plays no role in these exchanges. These results confirm the conclusions already published that sulphonamides can act directly on the cellular membrane as specific inhibitors of anion transport.

3. There is a correlation between the chemical structure of the sulphonamides and their capacity for inhibiting transmembrane anionic exchange. It is of significance that *N*-sulphamyl substitution, which abolishes the carbonic anhydrase inhibitory potency, does not destroy anionic inhibitory capacity and may even increase it.

4. For each sulphonamide the capacities for inhibiting chloride transport and oxalate transport are strictly identical. Inhibition appears non-competitive.

5. The temperature sensitivity of oxalate self-exchange is exactly the same as that of chloride self-exchange. From this, and from the nature of their inhibition by sulphonamides, it is proposed that chloride and organic anions share the same transport mechanism.

6. In the light of the present results the chloruretic action of sulphonamides in various tissues, in particular the kidney, is discussed.

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INTRODUCTION

The transmembrane exchanges of bicarbonate with chloride (HCO_3^-/Cl^-) or with organic anions (HCO_3^-/A^-) in red blood cells are inhibited by sulphonamides. Generally this effect is considered to be due to the inhibiting action of these compounds on intracellular carbonic anhydrase: all activity changes of the enzyme would modify the amount of bicarbonate available for exchange (Jacobs & Stewart, 1942; Keilin & Mann 1941; Maren & Wiley, 1970; Deuticke, 1972). In a recent paper (Cousin, Motais & Sola 1975) it has been demonstrated that sulphonamides can inhibit Cl^-/HCO_3^- exchanges by directly interfering with the transmembrane exchange process. On the basis of structural considerations it was proposed that bicarbonate shares the transport system which allows organic anions to permeate the red cell membrane of which the characteristics have been defined previously (Aubert & Motais, 1975) and that sulphonamides act as inhibitor on the 'carrier'. The expected consequence of this assumption is that organic anion self exchanges (A^-/A^-) should also be inhibited by sulphonamides. The present work was first undertaken to test this possibility. Using oxalate as prototypical or organic anions, it was shown that sulphonamides indeed act as inhibitors of the anion transport system.

The possibility that chloride may penetrate the red cell membrane completely or predominantly by a pathway which is not accessible to organic anions is actually open. The purpose of the second part of this paper was to investigate whether sulphonamides also inhibit chloride self-exchanges, as would be expected if the transports of chloride and organic anions have features in common.

A preliminary account of a part of these results has been published (Motais, Cousin & Sola, 1975).

METHODS

All experiments reported were performed by determining the rate of tracer efflux from radioactively labelled ox red cells, under steady-state conditions of anion concentrations.

Cell preparation

Blood collected into heparin by exsanguination was immediately transported to the laboratory and stored at 2° C. Before use, the cells were separated by centrifugation and plasma and buffy coat removed by aspiration; they were then washed three times in the washing medium and used as described below.

Labelling. After the washing procedure, the cells were resuspended in the electrolyte medium to be used for the experiment at a haematocrit of 50%; radioactive isotope was added and the suspension was incubated at 20° C for 2 hr (pH adjustment was 7·29 at 20° C giving, with the haematocrit used, pH 7·40 at 0° C, the temperature employed for efflux experiments). After centrifugation (10 min, 48,000 *g*), the supernatant was put aside for the determination of specific activity.

The labelled packed cells were then distributed in different batches, each of them being resuspended (haematocrit 50 %) in the incubation medium at exactly the same specific activity as at the end of the loading time, with or without the tested sulphonamide (10^{-3} M). After a night's incubation at 0° C to allow the drug to equilibrate fully with the cells, the suspension was centrifuged (refrigerated centrifuge, 10 min at 48,000 *g*). The supernatant was put aside to control the specific activity. The trapped extracellular volume, estimated with [^{14}C]inulin, is 5 % (w/w).

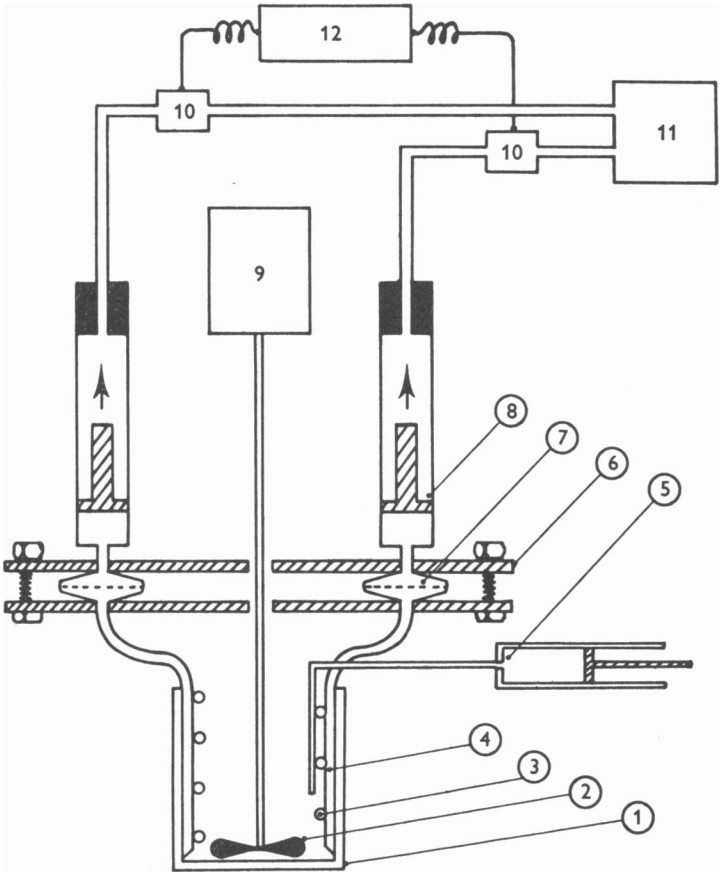


Fig. 1. Diagram of the automatic device for filtration: (1) to (9), filtration unit; (10) to (12), automatic control unit. The labelled packet cells are injected by a syringe (5) into the experimental medium contained in a beaker (1); the solution is stirred by a screw (2) driven by a motor (9); the plastic tubes for aspiration (4) are fixed by an inox spring (3). The aspiring syringes (8) are fixed on a circular holder (6) in which filter holders (7) are sandwiched. The opening of the Electrovan (10), which creates a negative pressure at the top of the sucking syringes, is piloted by the programmer (12). Just before starting the experiment a positive pressure is established to clear the sucking tubes of any experimental solution. Positive and negative pressures are produced by a pump (11).

Determination of the rate of tracer efflux

The appropriate electrolyte media were incubated at 0° C ± 0·01 (pH 7·4). At the start of the experiment, packed labelled cells at 0° C were injected into the medium (haematocrit 1 %) which was vigorously stirred by a small screw driven by a motor (1500 rev/min). The efflux of radioactive anion was followed by removing serial samples of cell-free supernatant by filtration of the suspension. The filtration technique was modified from Dalmark & Wieth (1972) to render it automatic, but the filtration material is the same as proposed by these authors. The automatic device, which is shown in Fig. 1, has the advantage of giving duplicate, cell-free samples at time intervals as short as 1 or 2 sec with a constant time for sucking; moreover the control of temperature can be very accurate, filtration being performed in a refrigerator. Because of the great temperature sensitivity of the chloride self-exchanges (according to Dalmark & Wieth, Q_{10} is about 8), the temperature of the external medium in the beaker was carefully controlled being exactly 0° C ± 0·01 at the start of the experiment. For that, the following procedure was adopted: in one side of the refrigerator the previously prepared filtration units were cooled and stored at −1° C; the flasks of experimental media were kept separately at 0° C ± 0·01 in a special refrigerated water-bath; before beginning an experiment, the filtration unit was quickly transferred to the other side of the refrigerator (temperature −1° C) where the experiment with the automatic device was performed; the beaker was immersed in a water-bath connected with an independent and precise temperature-control system. The experimental medium was rapidly introduced into the beaker through a freeze funnel. The experiment started automatically when the temperature in the beaker was exactly 0° C and the mixing motor was stopped 3 sec after starting to avoid an increase in temperature.

Determination of radioactivity. ^{36}Cl and [^{14}C]oxalic acid were obtained from the Centre d'Etudes Nucléaires de Saclay. Radioactivity in the filtrate was measured in a liquid scintillation spectrometer (Nuclear Chicago): after deproteinization with perchloric acid (final concentration: 6 %), a 1 ml. supernatant was added to 8 ml. Bray solution.

Calculation of the rate constant

In such a two compartment system in which the external one is very large in comparison with the intracellular one (haematocrit 1 %), the rate coefficient of anion exchange is obtained from the slope of the linear relation between

$$\ln\left(\frac{Q_\infty - Q_t}{Q_\infty - Q_0}\right)$$

and time t (Motais, 1967; Dalmark & Wieth, 1972). Q_0, Q_t and Q_∞ are the concentrations of isotope in the external medium at times 0, t and at isotopic equilibrium respectively.

Solutions

The medium used for washing incubation and chloride efflux experiments had the following composition (mM): 150, NaCl; 10, KCl; 20, Tris-HCl; pH = 7·4.

The medium used for incubation and efflux measurements in oxalate experiments had the following composition (mM): 140, NaCl; 10, KCl; 10, sodium oxalate; 20, Tris–HCl; pH = 7·4.

Sulphonamides (10^{-3} M) were dissolved at alkaline pH and pH re-adjustment was made immediately.

Drugs

Drugs were obtained as powder form: American Cyanamid Company (the Cl series), Hoescht (fursemide), Nativele (desamide), Theraplix (Chlorothiazide, hydrochlorothiazide), Geigy (Chlorthalidone), Leo (hydroflumethiazide), Robert et Carrière (Ambuside), Dr Kannan (AM sulf).

RESULTS

Oxalate self-exchanges

The temperature dependence of oxalate self-exchanges

The effect of temperature on the rate of oxalate exchanges was studied by measuring the tracer efflux into the external medium (cf. composition of medium described in Methods section). The cells had been labelled with [^{14}C]oxalate in the medium (pH 7·4) at the experimental temperature and the experiments were performed under steady-state conditions with respect to the anion concentration. The rate of [^{14}C]oxalate efflux was studied over a temperature range 0–35° C. The temperature dependences of oxalate exchanges is shown in Fig. 2 as an Arrhenius plot of the data. It can be seen that the relation between the reciprocal absolute temperature and the natural logarithm of the rate coefficient of oxalate exchanges is linear over the whole 0–35° C range. The Arrhenius activation energy is 26 kcal/mole. Thus the temperature dependence of the oxalate permeation process is very high, about twice the activation energy for salicylate (13 kcal/mole), the only organic acid previously studied from this point of view (Dalmark & Wieth, 1972). However it should be noted that it is the undissociated acid, and not the salicylate anion, which is transported through the red cell membrane (Dalmark & Wieth, 1972; Deuticke, 1972; Aubert & Motais, 1975).

Effect of sulphonamides on oxalate self exchanges

Using the filtration method of Dalmark & Wieth (1972), the rate of tracer efflux from red cells can be determined with a high degree of precision when the half-time of the exchange process is > 2 sec. Thus the chloride flux is too rapid to be measured at room temperature: the experiments have to be carried out at a temperature near 0° C. The self-exchange of oxalate, on the other hand, can easily be measured at room temperature the rate coefficient is 1·96 min^{-1} at 35° C. For a study of the relative influences of sulphonamides on the oxalate and chloride exchanges, however, it is preferable to carry out the two series of experiments at the same temperature. All measurements were therefore made at $0° \pm 0{\cdot}01°$ C. The various sulphonamides were added to the experimental solutions to give a concentration of 10^{-3} M. As described in the section on Methods, the

red cells had been kept the previous night in the sulphonamide solution at this concentration.

Fig. 3 illustrates the influence of some sulphonamides on the rate of oxalate exchange: it can be seen that oxalate exchanges are drastically reduced by fursemide and 3-benzene sulphonamidophenol, to a lesser extent by Cl 11,366 and hydrochlorothiazide, and that acetazolamide acts as a very poor inhibitor.

All the data concerning the inhibitory effect on oxalate permeability of sulphonamides are shown in Table 1. It can be seen that: (1) all the sulphon-

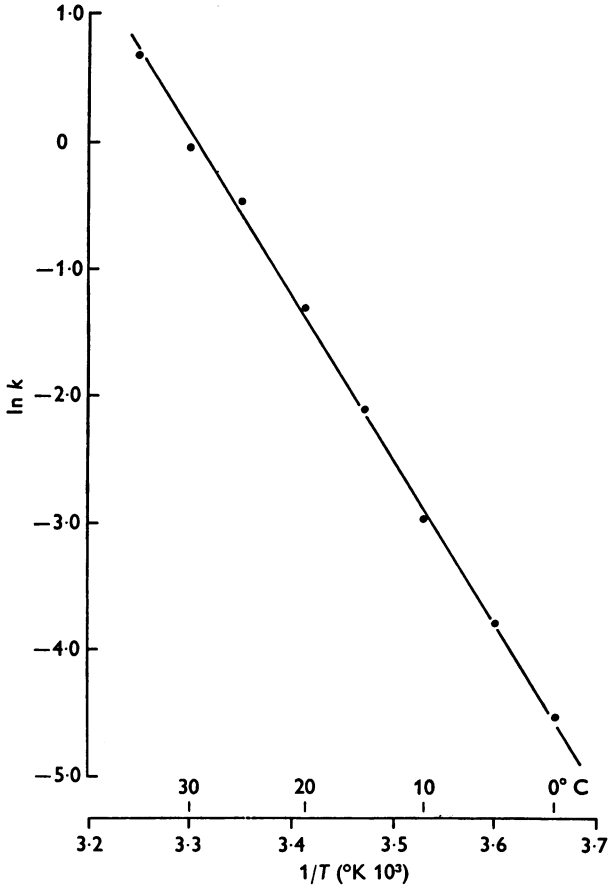


Fig. 2. Temperature dependence of oxalate steady-state exchange between ox red blood cells and a medium containing 10 mM oxalate (see composition in Methods); this Arrhenius diagram relates the natural logarithm of the rate coefficient of oxalate exchange (k) and the reciprocal absolute temperature ($1/T$). The values of the rate coefficient were 1·9611 min^{-1} at 35° and 0·011 min^{-1} at 0° C. The Arrhenius activation energy was 26 kcal/mole.

amides tested have inhibitory effects; (2) the substitution of a sulphamyl group on the nitrogen, which destroys the inhibitory action on carbonic anhydrase (Mann & Keilin, 1940; Krebs, 1948) does not reduce, in fact it may even increase, the anionic inhibitory potency (compare Compounds III and XVII, XXI and XXVI). One of the most active compounds in

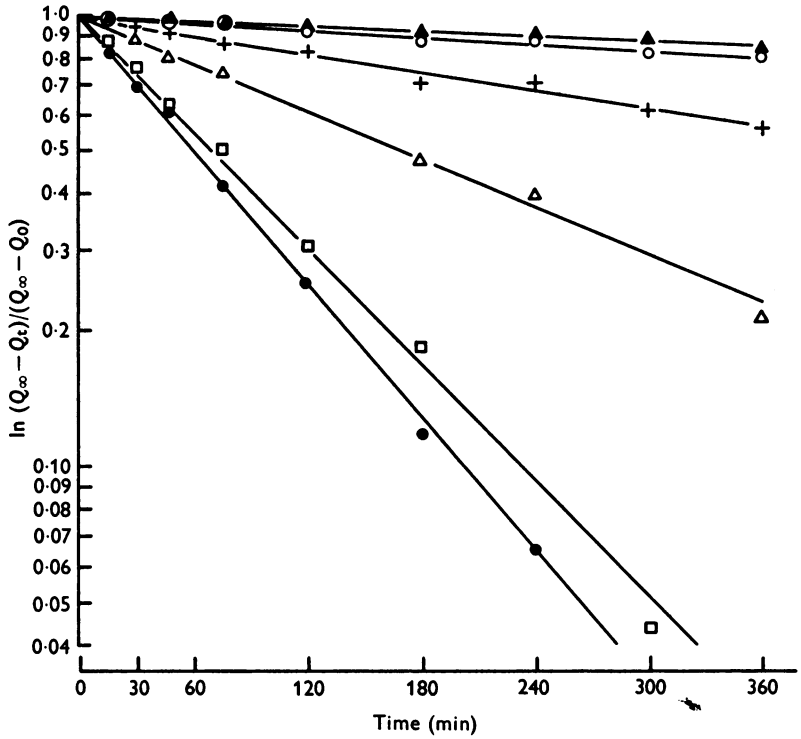


Fig. 3. The rate of steady-state oxalate exchange at 0° C, pH 7·40, between ox red cells and the external medium with or without sulphonamides (10^{-3} M). Red cells were pre-treated with the sulphonamide at the same concentration (see Methods). The rate coefficients given in Table 1 were calculated from the slopes of the graph of ln $(Q_\infty - Q_t)/Q_\infty - Q_0)$ *vs.* time (min) where Q_∞, Q_t, and Q_0, are the concentrations of isotope in the external medium at equilibrium, time t, and 0 respectively: ●, control; □, diamox; △, hydrochlorothiazide; +, benzolamide (Cl 11,366); ○, furosemide; ▲, 3 benzene sulphonamidophenol.

fact is a substituted sulphonamide (Compound XIX). There is thus no correlation between carbonic anhydrase inhibitory capacity and anionic inhibitory capacity. A study of Column 3 shows that an excellent carbonic anhydrase inhibitor may also be a good anionic (XXIV) or poor anionic (XXI, XXV) one; (3) there is no correlation between anionic inhibition

TABLE 1. Comparative inhibitory actions of various sulphonamides (10^{-3} M) on oxalate self-exchanges and chloride self-exchanges

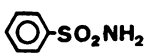
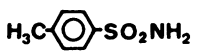
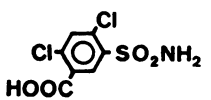
		2	3	4	5	6	7	8	9	10	11
						Oxalate exchange			Chloride exchange		
	Structure	Name or no.	Carbonic anhydrase (I_{50})	pK_a	Partition coefficient	Rate coefficient (min^{-1})	Relative rate	% inhibition	Rate coefficient (min^{-1})	Relative rate	% inhibition
		Control	—	—	—	$114\ 10^{-4}$	—	—	3·42	—	—
I	$H_2N\,SO_2NH_2$	Sulphamide	—	—	—	—	—	—	3·42	1·00	0
II		Benzene sulphonamide	$4·0\ 10^{-7}$ (*a*)	—	—	$87\ 10^{-4}$	0·76	24	2·87	0·84	16
III		Sulphanilamide	$1·3\ 10^{-5}$ (*b*)	10·4 (*e*)	0·15 (*e*)	$88\ 10^{-4}$	0·77	23	2·91	0·85	15
IV		*p*-nitrobenzene sulphonamide	—	—	—	$67\ 10^{-4}$	0·59	41	2·39	0·70	30
V		*p*-toluene sulphonamide	10^{-7} (*a*)	—		$72\ 10^{-4}$	0·63	37	2·39	0·70	30
VI		*p*-carboxybenzene sulphonamide	$4·4\ 10^{-6}$ (*b*)	—	—	$15\ 10^{-4}$	0·13	87	0·41	0·12	88
VII		2–4-dichloro-5-sulphamoylbenzoic	—	—	—	—	—	—	1·98	0·58	42

TABLE 1 (*cont.*)

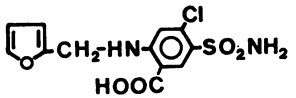
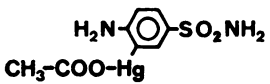
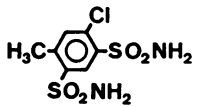
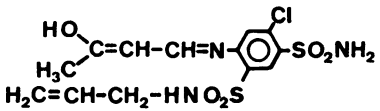
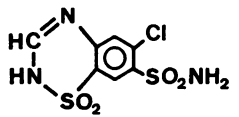
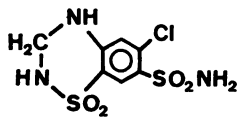
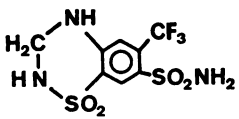
VIII		Furosemide	—	—	—	6 10^{-4}	0·05	95	0·14	0·04	96
IX		AM sulf	—	—	—	—	—	—	0·96	0·28	72*
X		Desamide (natirene)	—	—	—	23 10^{-4}	0·20	80	1·13	0·33	67
XI		Ambuside (hydrion)	—	—	—	—	—	—	1·74	0·51	49
XII		Chlorothiazide	2·2 10^{-6} (*c*)	6·7 (*c*)	0·04 (*c*)	—	—	—	1·27	0·37	63
XIII		Hydrochlorothiazide	2·3 10^{-5} (*b*)	8·8 (*e*)	0·20 (*e*)	39 10^{-4}	0·34	66	1·09	0·32	68
XIV		Hydroflumethiazide	1·7 10^{-4} (*b*)	—	—	—	—	—	1·20	0·35	65

TABLE 1 (*cont.*)

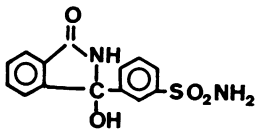
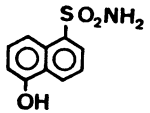
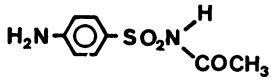
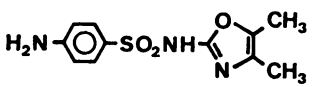
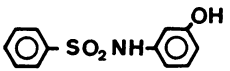
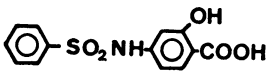
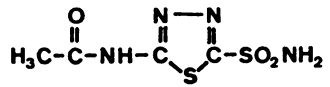
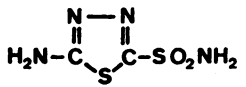
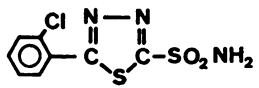
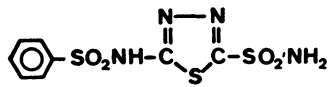
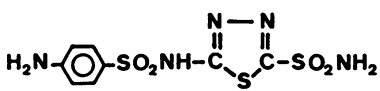
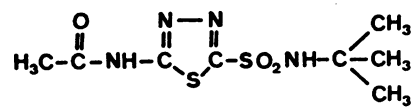
1		2	3	4	5	6	7	8	9	10	11
						Oxalate exchange			Chloride exchange		
	Structure	Name or no.	Carbonic anhydrase (I_{50})	pK_a	Partition coefficient	Rate coefficient (min^{-1})	Relative rate	% inhibition	Rate coefficient (min^{-1})	Relative rate	% inhibition
XV		Chlorthalidone	$3{\cdot}4\ 10^{-7}$ (*b*)	—	—	$66\ 10^{-4}$	0·58	42	2·53	0·74	26
XVI		5-hydroxy-1-napthalene sulphonamide	—	—	—	—	—	—	0·86	0·25	75
XVII		N^1-Sulphanyl-acetamide	$6{\cdot}0\ 10^{-4}$ (2)	—	—	$73\ 10^{-4}$	0·64	36	2·87	0·84	16
XVIII		N^1-(4,5-Dimethyl-oxazol-2-yl) Sulphanilamide	—	—	—	—	—	—	2·70	0·79	21
XIX		3-benzene sulphon-amidophenol	—	—	—	$4\ 10^{-4}$	0·04	96	0·14	0·04	96
XX		4-benzene sulphon-amido salicylic	—	—	—	—	—	—	0	0	100

TABLE 1 (*cont.*)

XXI		Acetazolamide (Diamox)	$1{\cdot}1\ 10^{-8}$ (*c*)	7·4 (*c*)	0·14 (*c*)	$98\ 10^{-4}$	0·86	14	3·42	1·00	0
XXII		Cl 5,343	$6{\cdot}0\ 10^{-8}$ (*d*)	7·8 (*d*)	—	$78\ 10^{-4}$	0·68	32	2·53	0·74	26
XXIII		Cl 13,580	$4{\cdot}8\ 10^{-9}$ (*c*)	6·6 (*c*)	79·00	—	—	—	1·20	0·35	65*
XXIV		Cl 11,366 (benzolamide)	$3{\cdot}6\ 10^{-9}$ (*c*)	3·2 (*c*)	0·001 (*c*)	$16\ 10^{-4}$	0·14	86	0·55	0·16	84
XXV		Cl 13,475	$4{\cdot}3\ 10^{-9}$ (*c*)	4·2 (*c*)	0·001 (*c*)	$78\ 10^{-4}$	0·68	32	2·39	0·70	30
XXVI		Cl 13,850	—	—	—	$71\ 10^{-4}$	0·62	38	2·57	0·75	25

Column 2. Numbers are the numerical designations used by the American Cyanamid Co.
Column 3. Molar concentrations for 50 % inhibition, I_{50}, of carbonic anhydrase (from differents publications).
Column 5. The ratio of concentrations in ether and phosphate buffered saline (from Wistrand, Rawls & Maren, 1960; Maren, 1963).
Columns 6 and 9. The absolute values of the rate coefficients (in min^{-1}) of oxolate and chloride self-exchanges.
Columns 7 and 10. The relative values (compared with controls) of oxalate and chloride self-exchanges.
Columns 8 and 11. Percentage inhibition relative to the control values, caused by the sulphonamides.
Data from the publications: (*a*) Krebs, 1948; (*b*) Beyer & Baer, 1961; (*c*) Wistrand *et al.* 1960; (*d*) Maren, Robinson, Palmer & Griffith, 1960; (*e*) Maren, 1963.
* Underestimated values because incomplete solubilization of the compound in aqueous solution.

and the pK_a of the compound. A substance with a low pK may be a good (XXIV) or a poor (XXV) inhibitor and similarly one with a high pK may be a poor (XXI, XXII) or fairly good (XIII) inhibitor; (4) there is no clear correlation between anionic inhibitory capacity and the liposolubility of the compound: compare XXIV and XXV, III and XIII.

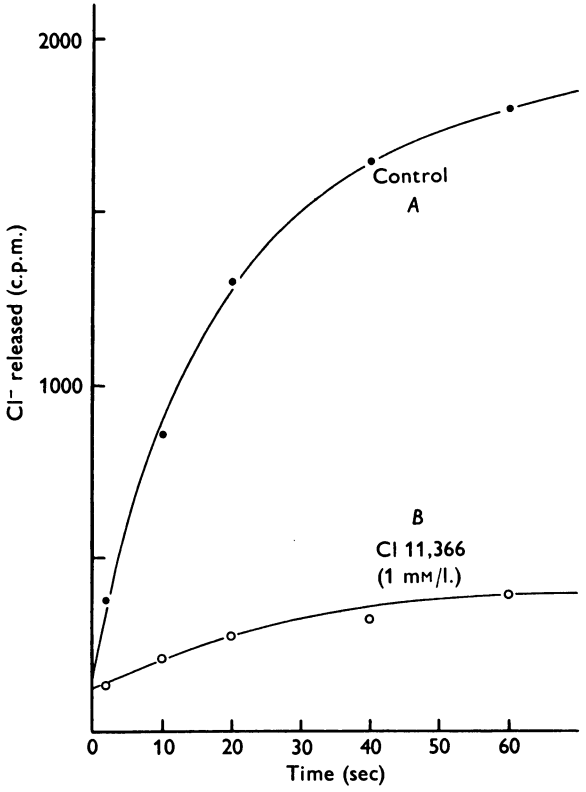


Fig. 4. Action of benzolamide (Cl 11,366) on chloride self-exchanges, Cl_o^-/Cl_i^-: ox red blood cells were washed and then equilibrated with a medium containing 160 mM-NaCl, labelled with ^{36}Cl. At time zero packed red cells were injected into a CO_2-free medium containing: 165 mM sodium gluconate (a very poor penetrating anion); 3 mM-NaCl; 10 mM Tris-gluconic acid; and with or without benzolamide (10^{-3} M) (pH 7·40; haematocrit 1% in the final suspension; temperature 20° C ± 0·05). Samples of the external medium were made by the filtration method using the automatic sampling device. The whole experiment was performed in a CO_2-free atmosphere. Ordinate, the radioactive chloride efflux expressed as the radioactivity of the external medium (in counts per minute per unit volume); abscissa, the time in sec. *A*, the pattern of the chloride efflux in the control experiment: *B*, the pattern of the chloride efflux in presence of benzolamide in the external medium.

Chloride self-exchanges

Evidence that sulphonamides can inhibit chloride self-exchanges

If red cells are suspended in a bicarbonate-free medium containing a slowly penetrating anion such as gluconate, chloride will leak out of the cell very slowly. Addition of a small amount of chloride to the external medium will induce a considerable increase of the rate of chloride efflux owing to extracellular, Cl_o, and intracellular, Cl_i, exchange, Cl_o/Cl_i. The pattern of such a chloride efflux forced by external chloride is illustrated in Fig. 4*A*. In the presence of benzolamide (Cl 11,366) at 10^{-3} M in the external medium, the chloride efflux is strongly reduced, demonstrating that this sulphonamide is able to inhibit Cl^-/Cl^- exchange.

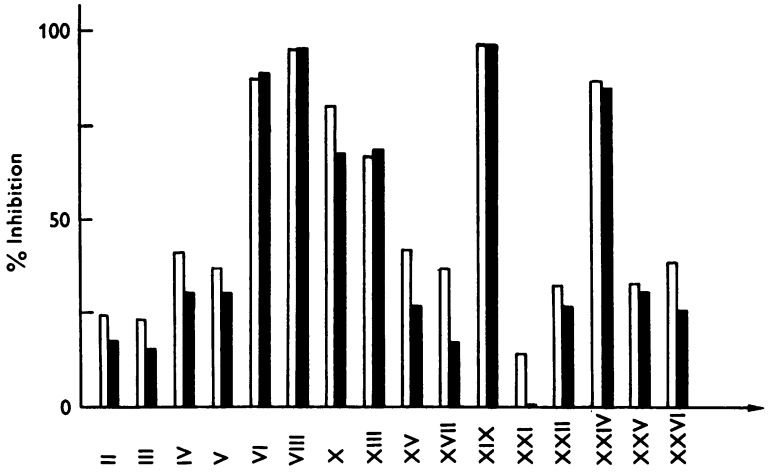


Fig. 5. Comparative inhibitory effect of sixteen sulphonamides on chloride and oxalate self-exchanges. Ordinate, % inhibition; abscissa, the numbers of compounds from Table 1; ■, chloride permeability; □, oxalate permeability.

Effect of sulphonamides on chloride self-exchanges

The inhibitory effect of sulphonamides on chloride self-exchanges was tested under the same steady-state conditions as for oxalate self-exchange (pH 7·40; temperature $0 \pm 0{\cdot}01°$ C; concentration of sulphonamide 10^{-3} M). The action of twenty-six sulphonamides was thus studied (all the compounds used for oxalate plus ten new ones). All the data are shown in Table 1.

The sulphonamides which were used in both series of experiments were found to have strikingly similar actions on chloride and oxalate permeabilities, as Fig. 5 clearly shows. The only small discrepancy appears for

acetazolamide which has a very poor inhibitory effect on oxalate self-exchange and none at all on that of chloride.

Thus all the conclusions already made regarding sulphonamide action on oxalate permeability are also valid for their action on chloride permeability. The data from the ten sulphonamides tested only on the chloride self-exchanges confirm the above conclusions: thus *N*-substituted sulphonamides may be excellent inhibitors (XX inhibits chloride permeability completely), and the substituted compounds are even more active than the

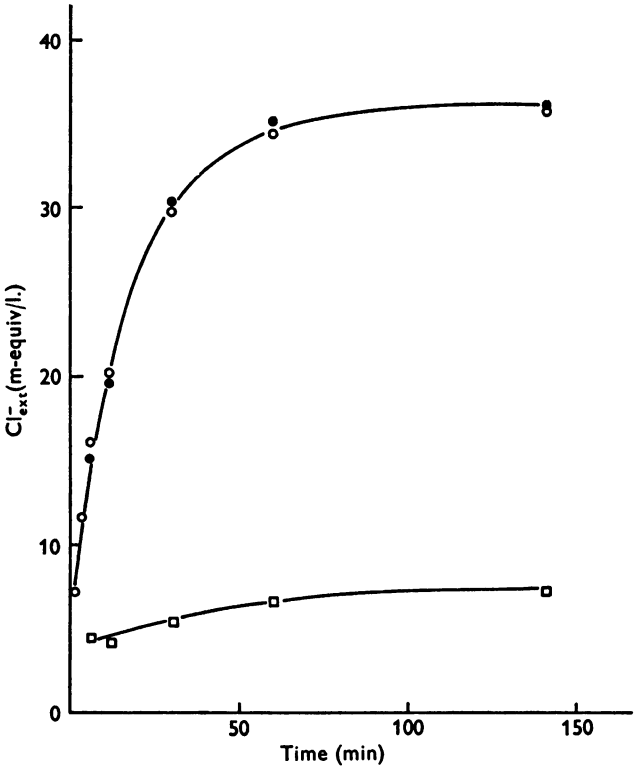


Fig. 6. Comparative effect of acetazolamide and SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid) on the exchange of extracellular pyruvate (160 mM) with cellular chloride: ●, control; ○, acetazolamide; □, SITS, a powerful inhibitor of anion exchanges in red cell (for references see Aubert & Motais, 1975).

parent drugs (compare III and XVIII). A poor carbonic anhydrase inhibitor such as XIV is as good an anionic inhibitor as XII, but a very good enzyme inhibitor (XXIII) is also a good anionic inhibitor. It should also be noted that all the thiazides (XII, XIII and XIV) have the same anionic inhibitory capacity.

We have previously shown that acetazolamide (Diamox), in opposition to all other carbionic anhydrase inhibitors tested (Cousin *et al.* 1975), does not act on the bicarbonate exchange process. In this work it is found that it is also a very poor inhibitor of organic anion self-exchanges and has no effect on Cl^-/Cl^- exchanges. Its lack of inhibition of the latter exchange has already been reported by Dalmark (1972). Like Deuticke (1972), we

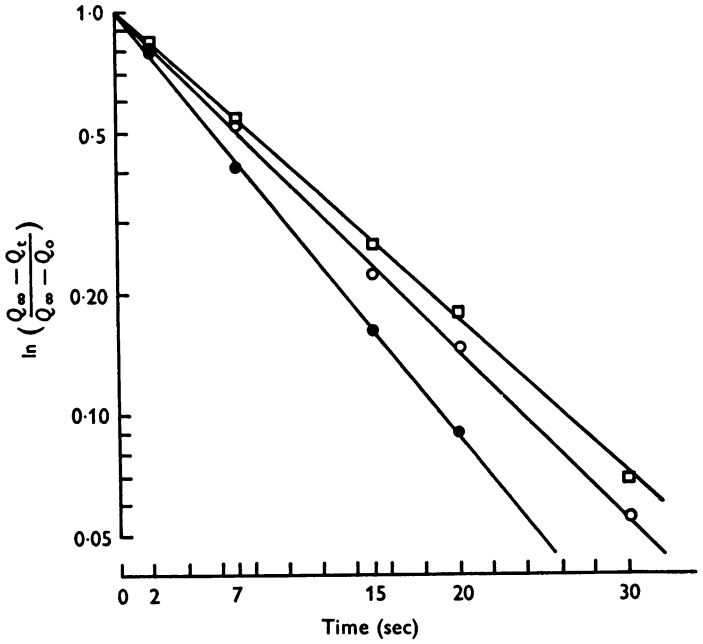


Fig. 7. Effect of acetazolamide on the steady-state chloride exchange at pH 8·7 (0° C). ●, control; ○, acetazolamide 10^{-3} M; □, acetazolamide 5×10^{-3} M. Q_∞, Q_t, and Q_0 are the concentrations of isotope in the external medium at equilibrium, time t, and zero time, respectively.

also found (Fig. 6) that Diamox, under our experimental conditions, does not significantly inhibit the $Cl_i^-A_o^-$ heteroexchanges. Since the pK of Diamox is 7·4 and the pH of the experimental solution for the self-exchange studies was 7·40 it is possible that the concentration of acetazolamide in active form was too low. Further experiments were therefore made at pH 8·7 using two concentrations of acetazolamide (10^{-3} M and 5×10^{-3} M). The results, given in Fig. 7, showed that under these conditions it apparently had an inhibitory effect on the chloride self-exchanges though nevertheless this effect was very slight, if significant.

DISCUSSION

The present study demonstrates that carbonic anhydrase inhibitors of the sulphonamide class inhibit anion permeability in ox red blood cells, the inhibitory potency depending on the structure of the compound; they also suggest that organic anions and chloride share a common pathway.

The inhibiting action of sulphonamides on anion permeability

Nature of the inhibition

Unsubstituted sulphonamides ($R\text{-}SO_2NH_2$) are specific inhibitors of carbonic anhydrase and the physiological responses to these substances have up to now always been interpreted as consequences of the enzyme inhibition. It has recently been shown that benzolamide (Cl 11,366) and Cl 13,580 inhibit the transmembrane Cl^-/HCO_3^- exchanges by acting directly at membrane level (Motais *et al.* 1975; Cousin *et al.* 1975). The present results show that the self-exchanges of organic anions (A_o^-/A_i^-) or chloride (Cl_o^-/Cl_i^-), in which carbonic anhydrase could not be involved, are also inhibited by sulphonamides. They thus clearly confirm that unsubstituted sulphonamides can act on systems other than carbonic anhydrase: in this case on the anion transfer mechanism of the red cell. It should be noted that the action is not one of a general modification of the membrane permeability because in the ox red cell fursemide, at a concentration of 10^{-3} M strongly inhibits anion transfer (95 % for oxalate, 100 % for chloride) without at all changing that of sodium (Motais & Sola, 1973).

As mentioned in the Introduction, this work was carried out to test the following hypothesis interpreting the nature of the interaction between sulphonamides and the transport mechanism. It has been shown that the entry of organic anions depends on a specific interaction of the penetrating anion with a component of the membrane. This interaction requires at least a three point attachment involving three oxygen atoms in the substrate which react with complementary loci on the membrane site to form ionic and hydrogen bonds (Aubert & Motais, 1975). Potentially such a three point attachment can be obtained with the bicarbonate ion and also with the sulphonamide group, SO_2NH_2, because the electronegativity of nitrogen is similar to that of oxygen. Consequently the demonstration that sulphonamides inhibit HCO_3^-/A^- and HCO_3^-/Cl^- exchanges by acting directly at the membrane level can be considered tentatively as a *competitive effect* of the sulphonamide on a common carrier shared by bicarbonate and organic anions (Cousin *et al.* 1975). At first sight the inhibitory effect of sulphonamides on the self-exchanges of organic anions (A^-/A^-) described in this

paper, support this hypothesis. A parallel study of sulphonamide action on chloride permeability, however, throws considerable doubt on this interpretation. In fact, as will be discussed below, it is probable that chloride and oxalate share a common transport mechanism yet their rate of penetration are very different: at 0° C the exchange rate coefficient is 3·4 min^{-1} for chloride and 0·008 min^{-1}, i.e. 500 times slower for oxalate. If sulphonamides act as competitive inhibitors, their inhibitory capacity for chloride should be very different from that for oxalate. Fig. 5 shows however that each sulphonamide inhibits the transports of chloride and oxalate to strictly the same extent. A non-competitive inhibition is a much more satisfactory explanation of these results.

Relationship between structure and inhibitory function in sulphonamides

It is important to keep in mind that there is no relation between the anionic transport inhibitory capacity of a sulphonamide and its carbonic anhydrase inhibitory capacity. This can be deduced by comparing Columns 3, 8 and 11 in Table 1, and has already been discussed above. Specific inhibitors of carbonic anhydrase have an unsubstituted SO_2NH_2 group and any substitution on the nitrogen destroys the activity (Mann & Keilin, 1940; Krebs, 1948). Such substitutions do not destroy the anionic inhibitory capacity however (see XVII, XVIII, XIX, XX, XXVI) and may in fact increase it (compare XXI and XXVI; III and XVII, XVIII; II and XIX, XX).

Considering a general relationship between structure and inhibitory capacity of the sulphonamides, the following points can be stressed.

(1) The substitution of various groups in the para position of the benzenesulphonamide structure influences potency: a COOH group in the para position considerably increases the inhibitory effect (VI) while the addition of another group (NH_2, NO_2 or CH_3) does not change the activity (III, IV, V).

(2) The position of the substitution with respect to the sulphanyl group influences potency. The addition of a COOH group in the meta position (VII) increases the activity less than when it is in the para position (VI). But if in addition to the COOH group in the meta position an apolar group is substituted in the para position, inhibition is very marked (VIII).

(3) The introduction of a second sulphanyl group meta to the first significantly increases anionic inhibitory potency (compare V and X), even if this sulphonamide group is included in a ring (chlorothiazide and congeners: XII, XIII, XIV).

(4) Among the thiadiazolsulphamides, substitutions at position 2 change the inhibitory potency considerably: Cl 5343 (XXII) with a two amino-substitution is a poor inhibitor; acetylation of this amino group strongly

reduces the inhibitory capacity (XXI); substitution of a benzene ring (XXIII) or a benzene sulphonamide (XXIV) on the other hand greatly increases it; the difference of activity between XXIV and XXV can only be explained by the presence of the NH_2 group for neither the pK nor the ether partition coefficient can account for it.

Evidence for a probable common pathway for organic anions and chloride

The results of the present work show that the chloride and oxalate transports have features in common. Two striking similarities should be noted: the Arrhenius activation energies are the same and the inhibitor sequences of sulphonamides are strictly identical.

The apparent activation energy for oxalate self-exchange was found to be 26 kcal/mole, i.e. exactly the same as that for chloride self-exchange (25·9 kcal/mole) also measured in bovine red cell by Wieth, Funder, Gunn & Brahm (1976).

Table 1 shows not only that all sulphonamides which inhibit oxalate transport also inhibit that of chloride, but also that the relative potencies of the various inhibitors are identical for the two transports. A study of compounds other than sulphonamides with inhibitory capacities has also demonstrated a complete parallelism between the inhibitions of chloride and oxalate transports (J. L. Cousin and R. Motais, unpublished).

That the two transports should be influenced in a strictly identical manner by a whole series of chemical substances and by temperature variation makes it reasonable to conclude that the rate of transfer of chloride and organic anions is controlled by the same structural membrane component. It should be noted that analogous reasoning leads to the conclusion that mono and divalent inorganic ions probably also share a common pathway (Dalmark & Wieth, 1972; Lepke & Passow, 1971).

Comparative aspects: the role of sulphonamides on the ionic permeabilities of different epithelia

The present results showing that unsubstituted sulphonamides can act in a specific fashion of an anion transport mechanism are of great interest with respect to the effects of sulphonamides on certain excretory processes.

Kidney. Sulphonamides are diuretic agents. The renal effect of some of them results from an inhibition of carbonic anhydrase. In contrast the diuretic effect of thiazides (compounds XII to XIV) and fursemide (VIII), which is characterized by excretion of sodium and chloride, is unrelated to carbonic anhydrase inhibition (Maren & Wiley, 1964). Their mode of action is still not clear. Generally it is believed (Golberg, 1973) that they act to inhibit active sodium transport across the epithelium of the renal

tubule. Recently, however, Burg, Stoner, Cardinal & Green (1973) showed that fursemide acts to inhibit, not active sodium but active chloride transport. The present study which demonstrates that fursemide is a good inhibitor of chloride transport in the ox red cell, supports Burg's conclusion. There is no experimental evidence at present indicating that thiazides and related compounds may also act by inhibiting chloride transport in kidney, as they do in red blood cells. However the structure–activity relationships established by Beyer & Baer (1961) in kidney are quite similar to the relationships between structure and chloride inhibitory function described above in red cells.

Cornea. Kitahara, Fox & Hogben (1967) and Candia (1973) have shown that sulphonamides depress chloride transport in frog cornea which is an epithelium lacking carbonic anhydrase. The similarity to the present results on the red blood cell suggests that the same mode of action could be involved.

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