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Inhibition of Anion Permeability by Amphiphilic Compounds in Human Red Cell: Evidence for an Interaction of Niflumic Acid with the Band 3 Protein

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Summary. In human erythrocyte, permeability to the anion is instantaneously, reversibly, and noncompetitively inhibited by the nonsteroidal anti-inflammatory drug, niflumic acid. The active form of this powerful inhibitor ($I_{50} = 6 \times 10^{-7}$ M) is the ionic form. We demonstrated that: (i) The binding of niflumic acid to the membrane of unsealed ghosts shows one saturable and one linear component over the concentration range studied. The saturable component vanishes when chloride transport is fully inhibited by covalently bound 4-acetamido-4′-isothiocyano stilbene-2,2′-disulfonic acid (SITS). Our estimate of these SITS protectable niflumate binding sites (about 9×10^5 per cell) agrees with the number of protein molecules per cell in band 3. These sites are half-saturated with 10^{-6} M niflumic acid, a concentration very close to I_{50}. (ii) Niflumic acid inhibits the binding reaction of SITS with anion controlling transport sites. These results indicate that niflumic acid and SITS are mutually exclusive inhibitors, suggesting that niflumic acid interacts with the protein in band 3.

Niflumic acid also decreases glucose and ouabain-insensitive sodium permeabilities. However, these effects are produced at a very high concentration of niflumic acid (in millimolar range), suggesting unspecific action, possibly through lipid phase.

In human red blood cell the anion transport seems to be mediated by an intrinsic membrane protein (i.e., band 3 protein) (Ho & Guidotti, 1975; Rothstein, Cabantchick & Knauf, 1976; Passow *et al.*, 1977). Anion exchange is selectively inhibited by the potent amino reagents isothiocyano stilbene disulfonic derivatives which irreversibly bind to this protein (Cabantchick & Rothstein, 1974; Zaki *et al.*, 1975).

The anion transport has also been shown to be inhibited by various chemically unrelated compounds (Deuticke, 1967; Schnell & Passow, 1969; Wieth, 1970; Gunn & Tosteson, 1971; Schnell, 1972). The only common property which can be found between these compounds is their

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amphiphilic character. These inhibitors are completely reversible and are not therefore suited for labelling of their sites of action. Structure activity studies performed with homogenous series of congeners has shown the importance of certain physico-chemical properties of the molecules in their inhibitory potency (Motais & Cousin 1977*a*, *b*; Motais, Sola & Cousin, 1978; Cousin & Motais, 1978*a*, *b*) but the mode of action of these inhibitors is not yet known. Several hypotheses have been proposed:

1) Because of the asymmetry of membrane lipid distribution between the inner and outer surfaces, the charged drugs are believed to bind differentially to the two membrane layers, thereby inducing asymmetrical expansion of the membrane (Seeman, 1972; Sheetz & Singer, 1974). Such an asymmetrical expansion of the lipid bilayer may disrupt the required structural organization of protein involved in transport. One of the difficulties with this explanation is that many agents produce changes in the membrane morphology of erythrocytes without affecting anion transport (Deuticke, 1970).

2) Hydrophobic adsorption of either anionic drugs to the lipids (McLaughlin, 1973; Fortes & Hoffman, 1974) or drugs which possess a large dipole moment (Andersen *et al.*, 1976) would produce a modification of the interfacial potential, thus modifying the relative concentration of anion and cation at the membrane interface. As a corollary of this hypothesis, the cation transport should be stimulated in the presence of these drugs. It has been shown that ANS,[1] an anionic drug which reversibly binds to erythrocyte membrane, decreases the anion exchange (Fortes & Hoffman, 1974), but instead of increasing the cation movements decreases them (Fortes & Ellory, 1975).

3) It is also possible that the drugs interact directly with the protein involved in transport.

The present work is concerned with an attempt to specify the mode of action of anionic amphiphilic compounds. We have chosen niflumic acid, which we show to be among the most powerful reversible inhibitors of anion permeability in red cells. The drug interacts with the band 3 protein but neither at the transport site nor at the modifier site (the two sites suggested by Dalmark, 1976). We show also that niflumic acid is relatively specific for anion transport even though glucose and cation

1 *Abbreviations:* SITS, 4-acetamido-4′-isothiocyano stilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid: H_2 DIDS, dihydro analog of DIDS; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid; ANS, 1-anilino-8-naphthalenesulfonic acid; PCMBS, *p* chloromercuriphenylsulfonic acid.

transports are inhibited by high drug concentrations. The mode of action is discussed.

Materials and Methods

Preparation of Cells

Freshly withdrawn or recently outdated human blood (collected into citrate dextrose solution) was obtained from the blood bank and stored at 4 °C. Bovine blood was collected into heparin by exsanguination and immediately transported to the laboratory and stored at 4 °C. Before use the red cells were separated by centrifugation and plasma and buffy coat removed by aspiration; they were then washed three times in appropriate buffer and used as described below.

Preparation of Human Resealed Ghosts

The technique for preparing uniform populations of human resealed ghosts was exactly as that described by Funder and Wieth (1976).

Flux Measurements

All the fluxes were performed at Donnan equilibrium.

^{36}Chloride Effluxes

The chloride self-exchange fluxes in intact cells were measured at pH 7.4, 0 °C. Labelling of cells with ^{36}Cl, isolation of labelled cells, determination of radioactivity in cell-free medium, and automatic technic of flux measurement have previously been described (Cousin & Motais, 1976). However, in the present work the hematocrit was 0.5 %. The composition of the solution used was: 150 mM NaCl, 10 mM KCl, 20 mM Tris, 10 mM D(+) glucose. In experiments with inhibitors, the cells were not incubated with them prior to the flux measurement; the drugs were only present in the experimental media at suitable concentration. The solutions used in experiments with ox red cells did not contain D(+)glucose.

The experiments with resealed ghosts were performed in the same way with the exception that the external solution contained 165 mM KCl, 2 mM Tris, and the pH was 7.2 (instead of 7.4 for intact cells). Because the volume of ghosts is chloride concentration dependent, in experiments where the chloride concentration changed, the quantity of labelled ghosts injected into radioactive chloride-free solutions was varied in order to always have the same concentration of ghosts in the media and consequently maintain the same ratio of concentration of binding sites to concentration of inhibitor.

^{14}Glucose Effluxes

The glucose effluxes were performed under the same conditions as chloride effluxes in order to compare the effects of drugs on both transport systems.

Human red cells were washed several times in saline buffer of the following composition: 140 mM NaCl, 20 mM Tris, 80 mM D(+)glucose at pH 7.4. Between each centrifugation, the suspension was kept at room temperature for a few minutes to allow equilibration of glucose between medium and cell water, the later incubation was performed at 0 °C, pH 7.4. The cells were then packed to a hematocrit of 80 %. ^{14}C glucose (Centre d'Etude Nucléaire de Saclay, France) was added to the cell suspension and allowed to equilibrate for more than ten half times of the subsequent efflux at 0 °C. At the start of efflux measurement 300 µl of labelled cell suspension were injected into 50 ml of vigorously stirred cold saline glucose medium (final hematocrit 0.5 %, pH 7.4; 0 °C); the efflux of ^{14}C glucose from cells to the medium was followed by serially isolating cell-free medium from the cell suspension by rapid filtration as described by Dalmark and Wieth (1972). Radioactivity of a constant volume of filtrate was measured.

^{22}Na Effluxes

Washed human red cells were resuspended in saline buffer (Hematocrit 50 %; pH 7.4, 37 °C) and ^{22}Na (Centre d'Etude Nucléaire de Saclay, France) was added. After an equilibration period the cells were washed with ice cold Na-free solution (100 mM $MgCl_2$, 30 mM sucrose, and 20 mM Tris; pH 7.4). The efflux experiments were started by injecting 250 µl of packed labeled cells into 50 ml of well-stirred medium (final hematrocrit 0.5 %; pH 7.4; 37 °C). Timed samples were withdrawn, centrifugated, and the radioactivity of a constant volume of supernatant was measured.

^{14}C Oxalate Effluxes

Human red cells were washed in saline buffer containing 140 mM NaCl, 10 mM oxalate, 10 mM glucose, and 20 mM Tris. The cells were suspended in oxalate medium at 10 % hematocrit and kept at 37 °C for 30 min. This operation was repeated twice in order to equilibrate internal and external medium with respect to oxalate. The cells were transferred at the temperature at which the fluxes were measured, titrated to pH 7.4, and loaded with radioactive oxalate (Centre d'Etude Nucléaire de Saclay, France).

Calculations

The kinetics were well described in the experiments by a two-compartment model with constant volume. The equation describing the time dependance of the specific activity in the cell-free medium is

$$Q_t = Q_\infty [1 - e^{-(k_o + k_i)t}]$$

wherein Q_t and Q_∞ are the specific activities at time t and isotopic equilibrium, respectively, the exponents k_0 and k_i are the rate coefficients for isotope efflux and influx, respectively.

In our experiments k_i could be neglected because the hematocrit was low (0.5 % vol/vol). The rate constant was calculated from the relation between $\ln(1 - Q_t/Q_\infty)$ and the time, t, by linear regression analysis. The slope of the graph was assumed to be equal to $-k_0$.

The fluxes per unit membrane area were calculated according to Funder and Wieth (1976) using the equation

$$j = k_0 \frac{V}{A} C_i$$

wherein k_0 (min^{-1}) is the rate constant of chloride self exchange, V (cm^3) the volume of water in cells or ghosts, A (cm^2) the membrane area and C_i (mole/cm^3) the cellular anion concentration.

Treatment of Cells by SITS

Basically, the method was that used by Ship *et al.* (1977) for DIDS and H_2DIDS binding experiments. Washed human red cells were resuspended at 10% hematocrit in saline medium (150 mM NaCl, 10 mM KCl, 10 mM HEPES) and allowed to react with SITS for specific times at 5 °C and pH 7.4. In experiments designed to measure the time course of the irreversible reaction (in the absence and in the presence of niflumic acid), the unreacted isothiocyanate was destroyed by addition of 2 mercaptoethanol as described in the procedure of Ship *et al.* (1977). The cells were then washed at 0 °C once in saline, twice in saline containing 0.5% albumin, and twice again with saline. In experiments in which niflumic acid was present during the incubation period, three additional washes were performed to remove all the drug. The cells were loaded with ^{36}Cl overnight and the rate of chloride efflux was determined for each group of cells.

Niflumic Acid Binding to Unsealed Ghosts

Ghosts prepared by the Dodge, Mitchell and Hanahan (1963) procedure, with three additional washes, were resuspended in saline buffer (150 mM NaCl; 10 mM KCl, and 10 mM HEPES, pH 7.4; 0 °C) at 1 mg/ml protein concentration (protein determinations were performed according to Lowry *et al.* (1951), using bovine serum albumin as a standard). One volume of ghost suspension was added to an equal volume of saline buffer containing various concentrations of niflumic acid and stored 10 min at 0 °C. (The final concentration of drug in the incubation medium was half of that in saline buffer and final protein concentration was 0.5 mg/ml). After the incubation period the suspension was centrifuged for 20 min at 0 °C (Sorvall RC2B rotor SS 34; 48,000 × g). The niflumic acid concentration was measured in the supernatant by optical density (OD) on an Aminco DW2 spectrophotometer at 288 nm (band width, 10 nm). At this wavelength the proteins absorb strongly. We measured the OD in supernatants in the absence of niflumic acid, and this value was substracted from all the OD values in the presence of the drug.

We also measured the binding of niflumic acid to ghosts obtained from cells treated with SITS and washed prior to the preparation of ghosts to remove unreacted SITS. The degree of inhibition of chloride self-exchange produced by covalently bound SITS was determined on aliquots of SITS-treated cells.

The membrane-bound niflumic acid was calculated from the total concentration and the free concentration (concentration remaining in the supernatant). The data were converted to numbers of molecules bound per cell, using 1.95×10^9 cells/mg of membrane protein as a constant factor which, according to Lepke *et al.* (1976), relates estimates of membrane protein to corresponding numbers of cells. The number of niflumic acid molecules bound per cell was plotted against the free drug concentration for normal ghosts and SITS-treated ghosts. At each concentration, the difference between the two curves gave the SITS protectable niflumic acid binding.

Determination of Lipophilic Character of Drugs

The hydrophobic properties of drugs were determined by thin-layer partition chromatography as previously described (Motais & Cousin, 1976*b*). Silica gel Silanised (60 HF 254, Merck) was the stationary phase, and mixtures of water and nonpolar solvent were the mobile phase. For a given compound, different R_f values were measured with different proportions of solvent and water in the mobile phase and the R_M value calculated according to the following equation (Bate-Smith & Westall, 1950):

$$R_M = \log\left(\frac{1}{R_f} - 1\right).$$

A particularly useful property of the R_M value, as contrasted with the R_f value, is that it is a simple function of the relative volumes of the solvent phases.

The R_M values were plotted *vs.* proportion of water in the mobile phase, the intercept of the straight line with the ordinate axis (determined by regression analysis) gave the R_M value for pure water ($R_{M_{H_2O}}$). $R_{M_{H_2O}}$ is a hydrophobic parameter equivalent to the logarithm of a partition coefficient.

Chemicals

The following reagents were used in these experiments: niflumic acid (UPSA Laboratories), meclofenamic acid (Parke Davis), phloretin (K and K laboratories), SITS (4 acetamido-4′ isothiocyano stilbene 2,2′ disulfonic acid) (British Drug House) HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Aldrich Chemicals Co.) bovine serum albumin fraction V (Sigma Chemical Co.) or ouabain (Sigma Chemical Co.). P-Chloromercuriphenyl sulfonic acid (Sigma Chemical Co). The methyl ester of niflumic acid was prepared by the small scale method of Schlenk and Gellerman (1960): 5.6 mg niflumic acid was dissolved in acetone. Diazomethane was bubbled in the solution until complete reaction. The product of the reaction gave a simple peak in gas chromatography and in a single spot in thin layer partition chromatography performed as described above.

Results

Characteristic of the Inhibition of Anion Self Exchange by Niflumic Acid

The self-exchange of radioactive chloride across the human red blood cells was measured in the presence of graded concentrations of niflumic acid at pH 7.4, 0 °C, hematocrit 0.5%. The results are shown in Fig. 1. The self-exchange flux was inhibited 50% by 6.3×10^{-7} M niflumic acid in the external medium ($I_{50} = 6.3 \times 10^{-7}$ M). Both the rapidity with which the drug affected the chloride self-exchange and the reversibility of the inhibition were shown by the experiments presented in Fig. 2. The chloride efflux from cells injected into tracer-free medium containing 5×10^{-6} M niflumic acid was compared to the control experiment (without

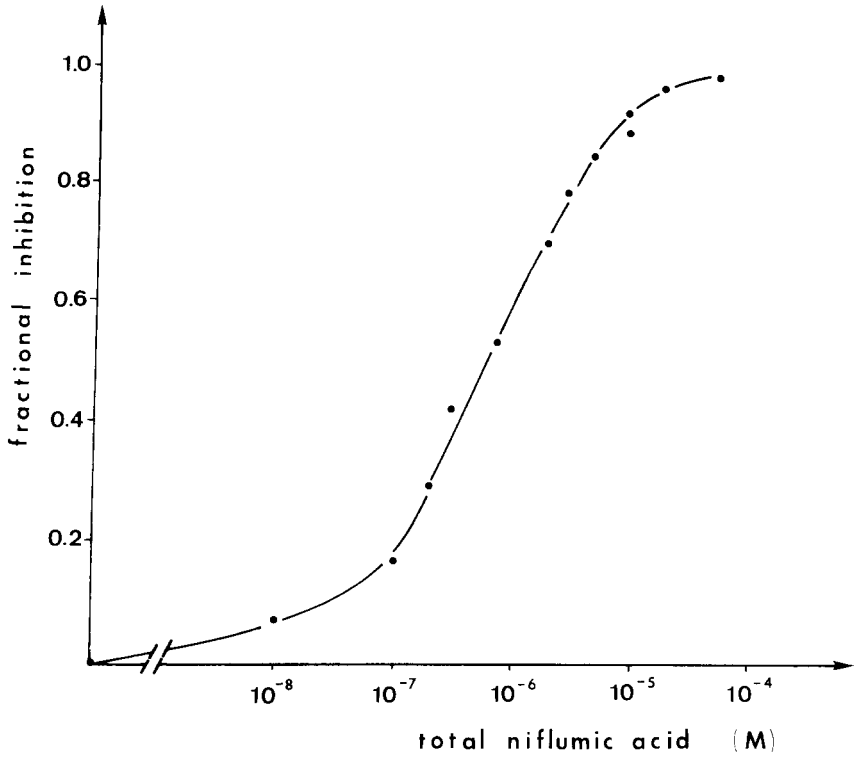


Fig. 1. Dose-response curve for niflumic acid inhibition of chloride self exchange in human erythrocytes. *Ordinate:* Fractional inhibition calculated from rate constants of chloride efflux in the presence and in the absence of inhibitor. *Abscissa:* niflumic acid concentration (molar) in logarithmic scale

niflumic acid). The efflux follows first order kinetics. This indicates that the level of inhibition was obtained within the 2 sec preceding the first sample. The reversibility of inhibition was demonstrated as follows: human red cells were (hematocrit 10%) suspended in the presence of 10^{-3} M niflumic acid for 2 hr at room temperature. The suspension was then washed six times in 20 volumes of drug-free medium before measuring the efflux rate. The rate constants for this experiment and for the control were not significantly different. The oxalate self-exchange was measured in the same conditions as the chloride self-exchange (pH 7.4, 0 °C, hematocrit 0.5%). The niflumic acid also inhibited this divalent organic anion exchange. Table 1 shows that the I_{50} values were similar for chloride and oxalate self exchanges in both human and bovine cells.

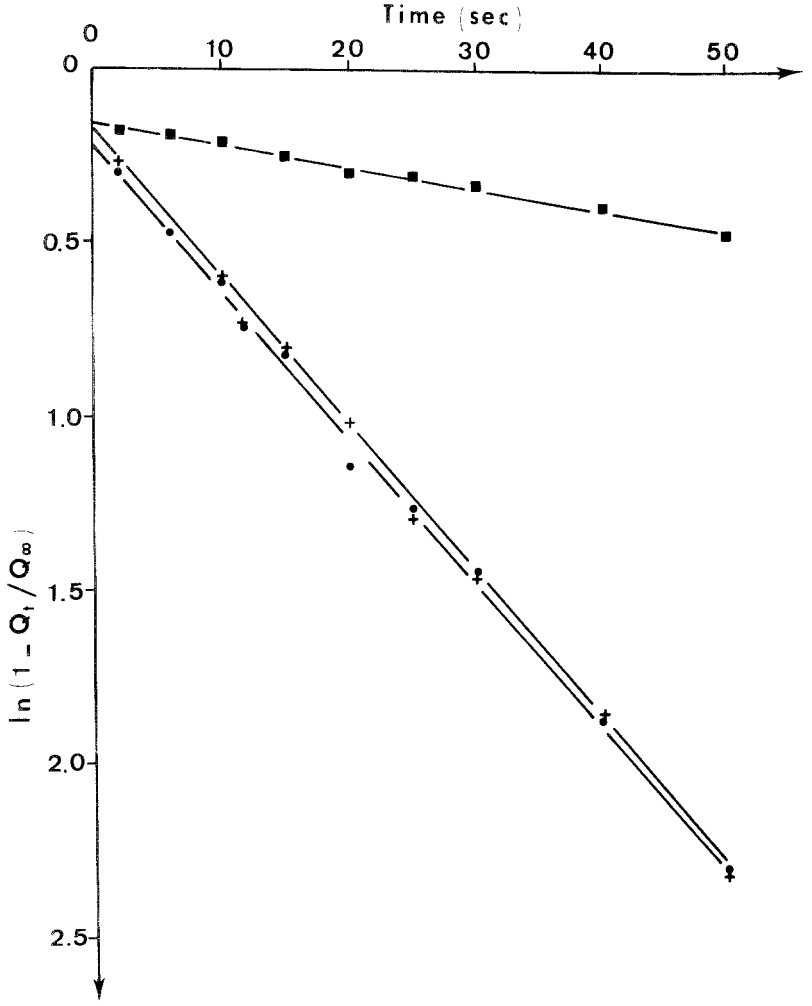


Fig. 2. Effects of niflumic acid on chloride self exchange in human red cells. *Ordinate:* natural logarithm of $1-Q_t/Q_\infty$. Q_t and Q_∞ are the concentrations of ^{36}Cl in external medium at time t and at isotopic equilibrium, respectively. *Abscissa:* time in seconds. (●) control cells; (+) cells treated with niflumic acid (10^{-3} M, hematocrit 10%) then washed in drug-free medium; (■) drug-free cells that were first exposed to niflumic acid (5×10^{-6} M) at the beginning of the efflux measurement

Table 1. Inhibitory potency of niflumic acid against chloride and oxalate self-exchanges in human and ox red cell[a]

	I_{50} (M) chloride self-exchange	I_{50} (M) oxalate self-exchange
Human red cells	6.3×10^{-7}	5×10^{-7}
Bovine red cells	2×10^{-7}	2×10^{-7}

[a] I_{50} is the drug's concentration required to produce 50% inhibition at 0 °C, pH 7.4.

Active Form of the Drug

The I_{50} value was the total concentration of the drug producing 50% inhibition of chloride flux. But the acidic drug could exist in the bulk phase as the ionized or unionized form depending on the pKa value and the pH of the medium. The pKa of niflumic acid is about 4.6 (*unpublished data*). The concentration of the uncharged form represents 0.2 and 4% of the total drug concentration at pH 7.2 and 6.0, respectively. Thus, for a given total concentration of drug, the concentration of charged form did not decrease significantly between pH 7.2 and 6, whereas the concentration of the neutral form increased by 2,000%. If the anionic form was active or if the two forms had the same inhibitory activity the total concentration reducing the control flux by 50% was expected to be identical at these two pH values. On the contrary, if the active form was the unionized form, the I_{50} value would be twenty times weaker at pH 6.0 than at pH 7.2.

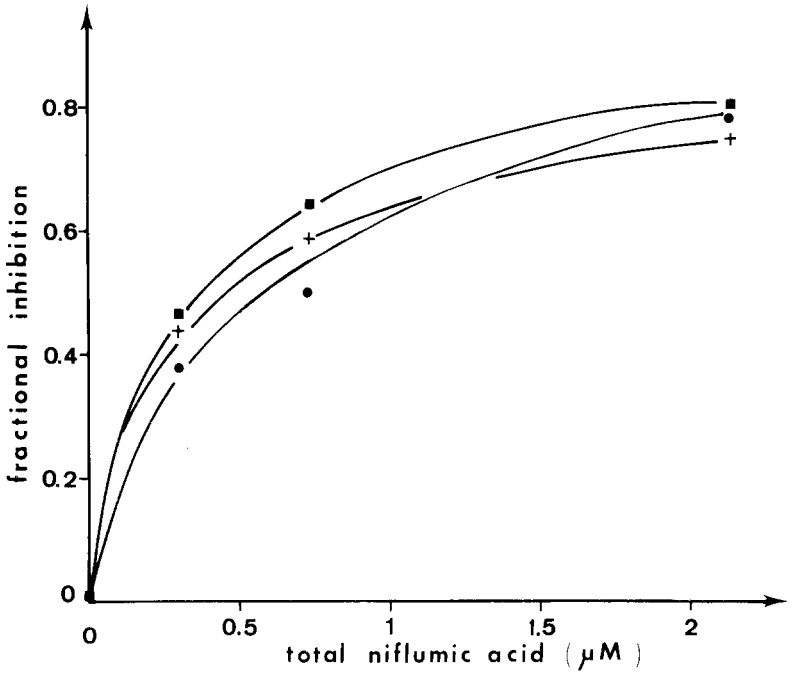
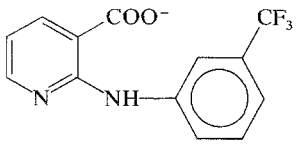
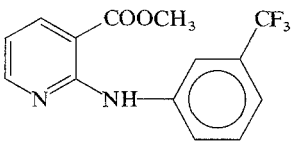


Fig. 3. Inhibition of chloride self-exchange in resealed ghosts as a function of niflumic acid concentration in external medium at three different pH values. (■) pH = 6.0; (+) pH = 6.5; (●) pH = 7.2. *Ordinate:* fractional inhibition of chloride self exchange. *Abscissa:* niflumic acid concentration (micromolar). The drug's concentration causing 50% inhibition (I_{50}) is the same at these three pH values

Table 2. Comparison between the inhibitory activity (I_{50}) and lipophilicity ($R_{M_{H_2O}}$) of niflumic acid and its methyl ester derivative

	
Niflumic acid	Niflumic acid methyl ester
$I_{50} = 6.3 \times 10^{-7}$ M	$I_{50} = 2.8 \times 10^{-5}$ M
$R_{M_{H_2O}} = 2.45$	$R_{M_{H_2O}} = 3.11$

The dependence of chloride self-exchange on the total niflumic acid concentration was measured at three pH values 7.2, 6.5, and 6.0 in red cell ghosts. Indeed, in ghosts, unlike in whole red cells, there was no shift in transmembrane potential with the variation of the external pH.

The fractional inhibition of chloride fluxes at different niflumic acid concentrations is shown in Fig. 3. There was no shift in the dose response curve with changing pH. Thus, the I_{50} value is constant at these three pH values, suggesting that: either the active form is the charged one or both forms are equally active. The latter solution is not supported by the following experiments.

We compared the inhibitory activity (I_{50}) of niflumic acid with its methyl ester derivative (Table 2).

The ester is 50-fold less active than niflumic acid as indicated by I_{50} values (2.8×10^{-5} M and 6.3×10^{-7} M, respectively). Thus, we conclude that the negatively charged molecule is the active form. Since the methyl ester derivative is a poor inhibitor, the uncharged form of niflumic acid probably also inhibits the chloride self-exchange flux but very poorly.

Binding of Niflumic Acid to the Membrane. Effect of the Covalently Bound SITS

The binding of niflumic acid to the human erythrocyte membrane exhibited one part saturable and one linear, unsaturable component over the concentration range studied (Fig. 4 *inset*, upper curve). We also performed binding experiments with membranes of cells pretreated by SITS as described in *Materials and Methods*. In our conditions SITS was covalently bound to such an extent that the chloride flux was fully inhibited. It can be seen (Fig. 4, lower curve) that the binding of niflumic

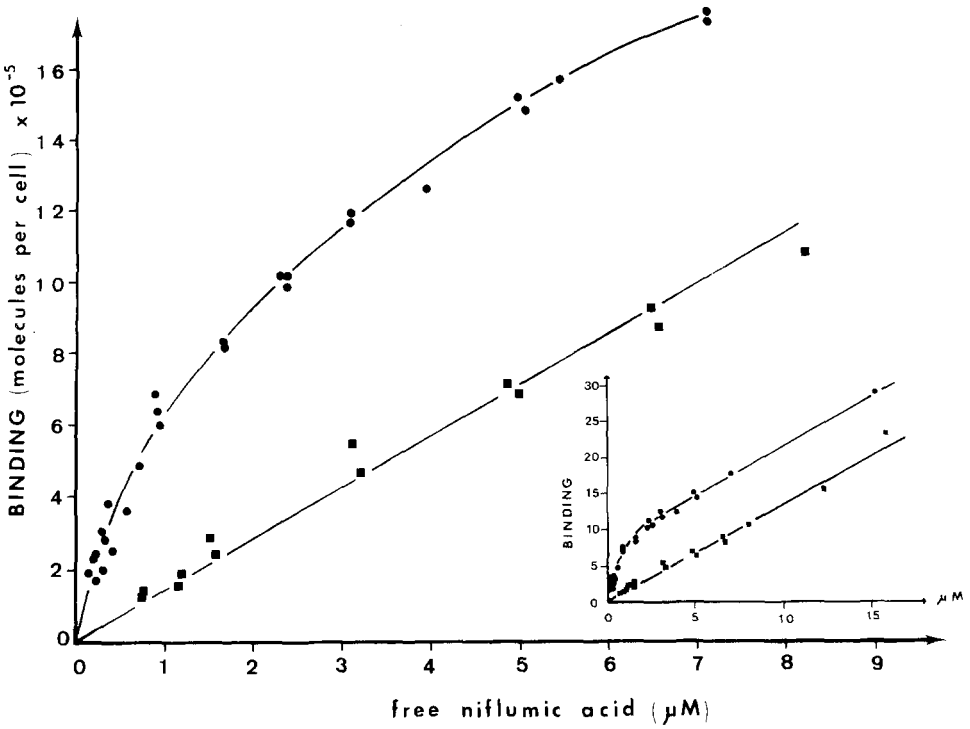


Fig. 4. Niflumic acid binding to hemoglobin-free unsealed ghosts. *Ordinate:* bound niflumic acid (10^5 molecules per equivalent cell). *Abscissa:* free drug's concentration in the medium (μM). (●) niflumic binding to ghosts from intact cells; (■) binding to red cells ghosts, the anion transport system of which was inhibited by covalently bound SITS. *Inset:* binding curves for all the concentration range tested

acid was considerably reduced after SITS treatment. This SITS-unprotectable niflumic acid binding was unsaturable. At each concentration the difference between the upper and the lower curves gave the number of SITS-protectable niflumic acid binding sites (Fig. 5). The data was also plotted according to the Scatchard method (Scatchard, 1949) (*inset*, Fig. 5). The intercept of the curve with the abscissa axis gave the number of SITS protectable niflumic acid binding sites. This number, 0.9×10^6 sites per cell, is very close to the number of band 3 copies per cell (about 10^6 per cell: Juliano, 1973; Steck, 1974). The dissociation constant (K_D) of the niflumic acid can be obtained from the negative of the slope, which is the reciprocal of K_D. K_D was 10^6 M, a value in relatively good agreement with the I_{50} for the inhibition of anion self exchange ($I_{50} = 4 \times 10^{-7}$ to 7×10^{-7} M). It will be noticed that I_{50} refers to a total drug concentration whereas K_D refers to a free drug concentration.

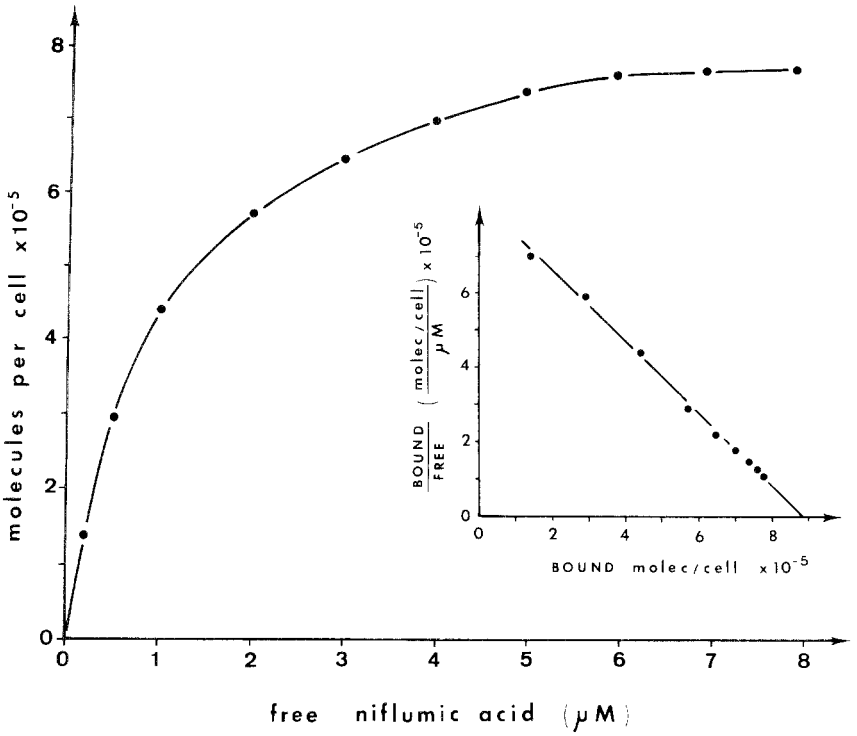


Fig. 5. SITS protectable niflumic acid binding sites. This curve was obtained by the difference between the upper and the lower curve in Fig. 4. *Inset:* the same data plotted by the Scatchard method; *Ordinate:* SITS protectable niflumic acid binding sites (10^5 molecules per equivalent cell) divided by free drug's concentration (μM). *Abscissa:* SITS protectable niflumic acid binding sites (10^5 molecules per equivalent cell)

Effect of Niflumic Acid on the Time Course of the SITS Binding

Previous studies of the effects of isothiocyanate derivatives of stilbene disulfonic acid on anion exchange suggested that irreversible fixation of these compounds to anion permeability controlling sites is preceded by reversible combination (Cabantchick & Rothstein, 1972). More recent studies showed that the irreversible fixation of H_2DIDS and DIDS on the membrane is relatively slow (Lepke *et al.*, 1976; Ship *et al.*, 1977) and that SO_4^{--} permeability is inhibited by both reversibly and irreversibly bound H_2DIDS (Lepke *et al.*, 1976). We measured the time course of irreversible SITS binding to red cell. The cells were incubated with 2×10^{-5} M of SITS for specified times at 5 °C. The reaction was stopped by addition of mercaptoethanol (Ship *et al.*, 1977). The cells were washed

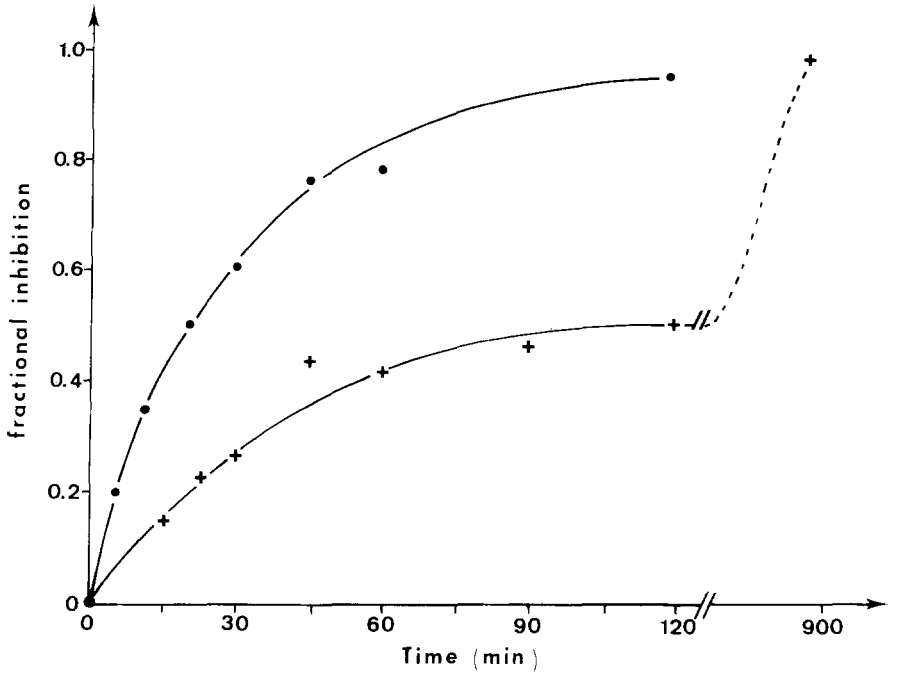


Fig. 6. Effect of niflumic acid on the time course of irreversible SITS binding to sites related to anion transport. Human red cells were incubated with SITS (2×10^{-5} M; hematocrit 10%; 5 °C) for specified time in the absence (●) or in the presence (+) of niflumic acid (2×10^{-4} M) in the medium. The reaction was stopped and the unreacted SITS removed by washing. For each group of cells fractional inhibition of chloride self-exchange (due to covalently bound SITS) was determined. *Ordinate:* fractional inhibition of chloride self exchange caused by irreversible SITS. *Abscissa:* incubation time (min)

with albumin and loaded with $^{36}Cl^-$. The rate of the chloride equilibrium flux was then measured in the absence of inhibitor in the medium. The fractional inhibition was assumed to represent the fractional occupancy of SITS binding sites related to anion transport. The results are shown in Fig. 6. In another experiment, the cells were incubated with SITS (2×10^{-5} M) in the presence of niflumic acid 2×10^{-4} M. At the end of the incubation period, the cells were washed as described in *Materials and Methods* in order to remove unreacted SITS and niflumic acid (it was reversible, as shown in Fig. 2). The chloride fluxes were measured without niflumic acid in the external medium. As can be observed, the rate of irreversible SITS binding is drastically decreased when the incubation of cells with SITS is performed in the presence of niflumic acid. Because the decrease in the rate of the reaction could be due to a

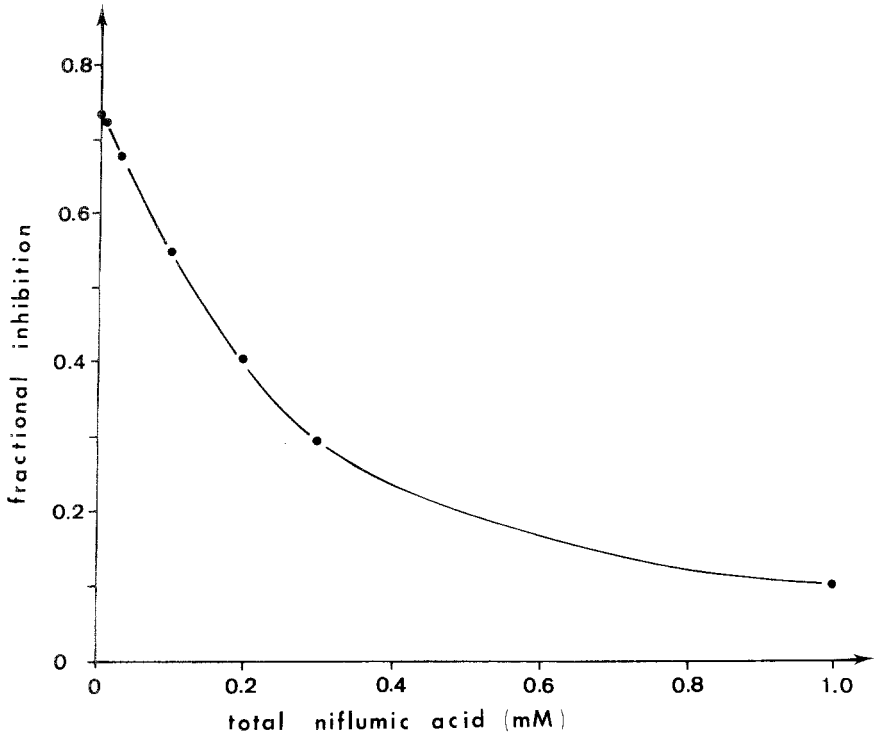


Fig. 7. Inhibition of irreversible SITS binding to sites related to anion transport by increasing niflumic acid concentration. The experimental conditions were the same as in Fig. 6 except that cells were incubated with SITS for 60 min in the presence of specified niflumic acid concentration. *Ordinate:* fractional inhibition of chloride exchange due to irreversibly bound SITS. *Abscissa:* niflumic acid concentration (mM)

chemical inactivation of SITS by niflumic acid, we performed the following experiment. The SITS was allowed to react with red cells in the presence of niflumic acid, 2×10^{-4} M, at 5 °C for 120 min (as in the previous experiment), and the suspension was transferred to 20 °C for 15 hr. The cells were washed, loaded, and the rate constant was measured as described above. The fractional inhibition was 0.97, indicating that SITS was always reactive, but the reaction was inhibited by niflumic acid. The decrease in the covalent reaction rate was niflumic acid concentration dependent as shown in Fig. 7. Cells were in contact with SITS for 60 min with a suitable niflumic acid concentration. The presence of 2.5×10^{-4} M of drug reduced SITS binding to 50% of the value in the absence of niflumic acid.

Effect of Chloride on Inhibition by Niflumic Acid

The dependence of chloride equilibrium flux on chloride concentration passes through a maximum in both red cells and ghosts (Cass & Dalmark, 1973; Funder & Wieth, 1976). This indicates the existence of two counteracting effects: the activating effect is related to saturation of the transport system; the inhibitory effect is believed to represent the binding of chloride to a modifier site since the affinities of chloride for the site and for the modifier site are different (67 and 335 mM, respectively; Dalmark, 1976). It is theoretically possible to determine the site of inhibition if the inhibitor competes with chloride for one of the two sites. For this purpose we prepared ghosts with various chloride concentrations and measured for each group the chloride efflux in the absence and in the presence of niflumic acid. The results are shown in Figs. 8 and 9. The upper curve in Fig. 8 shows that chloride flux in red cell ghosts passes through a maximum. That confirms previous results of

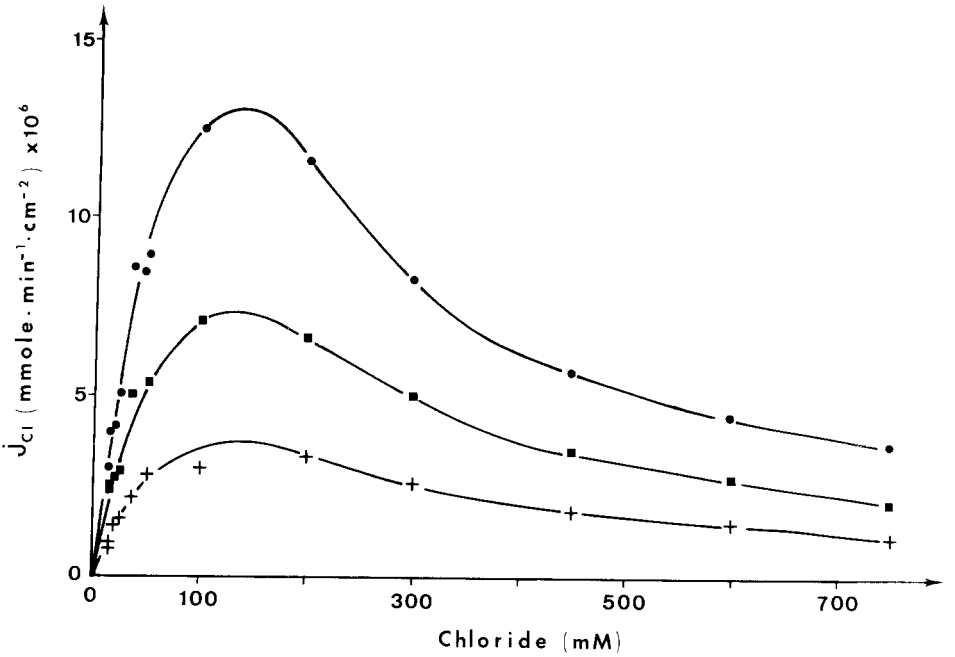


Fig. 8. Effect of increasing chloride concentration on chloride flux in the absence (●) and presence of 5×10^{-7} M (■) and 2×10^{-6} M (+) niflumic acid. *Ordinate:* chloride flux in 10^6 mmol · min^{-1} · cm^{-2}. *Abscissa:* chloride concentrations in mM. The fluxes were measured in human red cell ghosts at 0°, pH 7.2

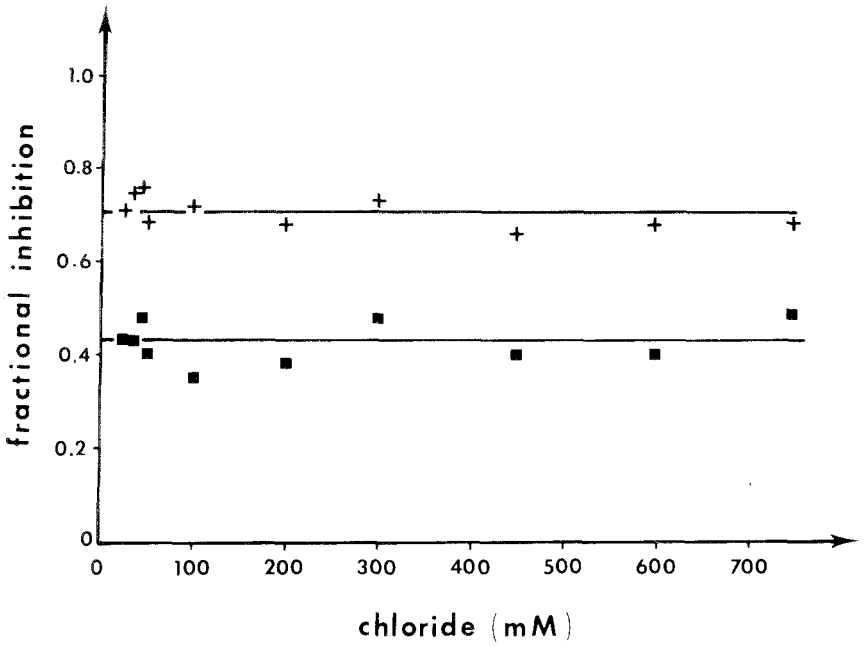


Fig. 9. Inhibition of chloride flux by 5×10^{-7} M (■) and 2×10^{-6} M (+) niflumic acid as a function of chloride concentration in red cell ghosts and medium. *Ordinate:* fractional inhibition. *Abscissa:* chloride concentration in mM

Funder and Wieth (1976). If measured in the presence of 5×10^{-7} and 2×10^{-6} M niflumic acid, the fluxes retain their characteristic features. Figure 9 shows that the fractional inhibition of chloride flux by niflumic acid is not affected by increasing chloride concentration from 15 to 750 mM. This indicates that niflumic acid inhibits the chloride self exchange in a noncompetitive manner. Its binding site is neither the transport site nor the modifier site.

Effect of Temperature on Inhibition by Niflumic Acid

Since the chloride self-exchange cannot be measured by the usual filtration technic at temperatures up to 15 °C, we performed this study on oxalate self-exchange which occurs much more slowly[2]. The temperature

2 Using oxalate as a test anion needs some justification. Indeed, in human red cells two transport systems have been reported for oxo and hydroxy substituted aliphatic monovalent organic anions such as pyruvate, lactate, and glycolate: the "classical" inorganic anion transport system and another different system (Halestrap, 1976; Rice & Steck, 1976; Deuticke, Rickart & Beyer, 1978).

dependence of oxalate self exchange in human red cells is shown in Fig. 10. The Arrhenius activation energy was constant over all the temperature range (0–30 °C) with a value of 31 kcal/mol. Similar values have been reported for inorganic mono and divalent anions in human (Passow, 1969; Dalmark & Wieth, 1972; Deuticke, 1970) and bovine red cell (Wieth *et al.*, 1974, Cousin & Motais, 1976).

The inhibition of oxalate self-exchange by niflumic acid was temperature dependent. The I_{50} value increased by a factor of 10 between 0 and 30 °C (Fig. 11). If one assumes that the variation of I_{50} with temperature represents the variation of equilibrium of the reaction between inhibitory site and niflumic acid with temperature, the enthalpy change associated with the site-inhibitor complex formation is given by the Van't Hoff equation:

$$\frac{d \ln K_A}{dt} = \frac{\Delta H}{RT^2}$$

where K_A is the association constant assumed to be $1/I_{50}$, ΔH the enthalpy change, T the absolute temperature, and R the gas constant. The plot of $\ln 1/I_{50}$ for niflumic acid against the reciprocal of the absolute temperature is shown in Fig. 11. The curve was linear, indicating that ΔH is constant over the temperature range under investigation. The molar free energy ΔG of site-inhibitor complex formation is given by:

$$\Delta G = -RT \ln 1/I_{50}$$

However, the following arguments (J.L. Cousin and R. Motais, *unpublished; see also* Motais, 1977) suggest that divalent organic anion oxalate crosses the human erythrocyte membrane exclusively via the inorganic anion transport system.

1) Pyruvate and lactate transfer is weakly sensitive to isothiocyano stilbene disulfonate derivatives and transfer can be observed when inorganic anion transport system is inhibited (Halestrap, 1976; Deuticke *et al.*, 1978). In contrast, irreversibly bound SITS induces exactly the same inhibitory pattern on oxalate and chloride self-exchange.

2) Lactate transfer is highly sensitive to certain SH reagents (particularly PCMBS) (Deuticke *et al.*, 1978). In marked contrast, oxalate self exchange is not affected by pretreatment of cells with PCMBS or when flux measurements are performed with PCMBS in external medium.

3) In addition, the dose-response curves for the inhibition of oxalate and chloride self exchange (measured in the same conditions) by various amphiphilic coumpounds are superimposed, and the I_{50} values are identical for all the compounds (*see* Table 1 for niflumic acid).

4) The Arrhenius activation energy curves show a break between 10 and 20 °C for pyruvate, lactate, and glycolate (Halestrap, 1976; Deuticke *et al.*, 1978). In contrast, activation energy for oxalate transfer is constant between 0 and 30 °C and close to the value observed for inorganic anions.

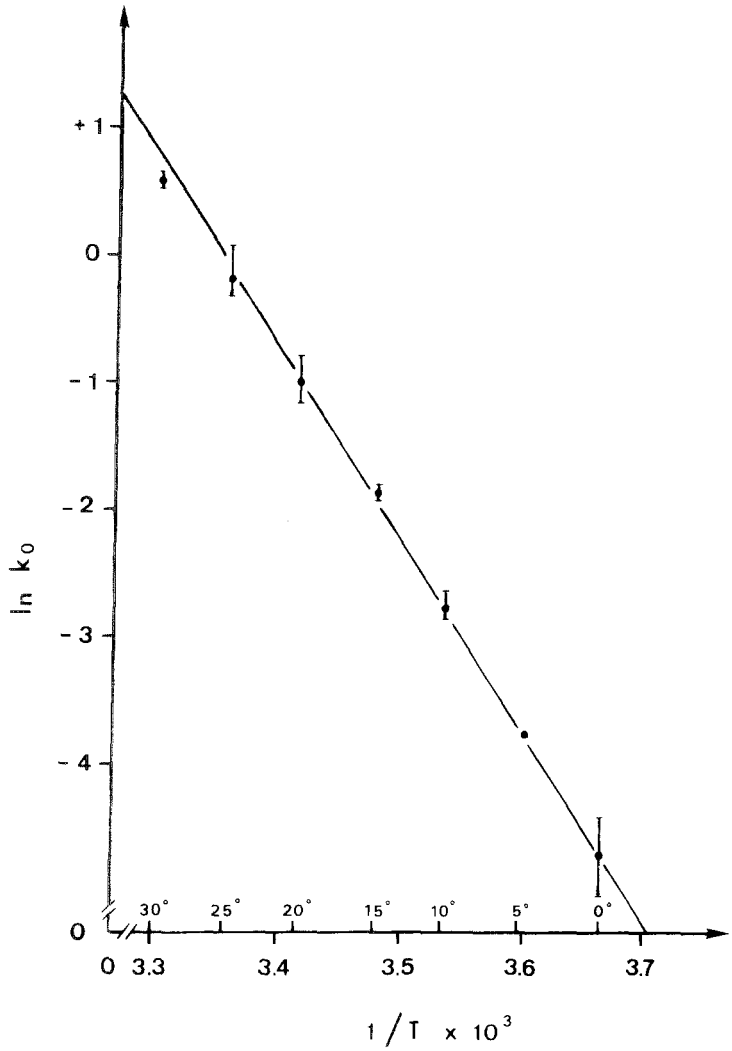


Fig. 10. Temperature dependence of oxalate steady-state exchange between human red cells and external medium containing 10 mM oxalate. Arrhenius plot. *Ordinate:* natural logarithm of the rate constants of oxalate exchange k_0. *Abscissa:* reciprocal absolute temperature ($1/T$). *Circles and bars:* respective mean and range of measurements obtained in several experiments. *Solid line:* straight line obtained by linear regression analysis

and the entropy, ΔS, can be calculated from

$$\Delta G = \Delta H - T\Delta S$$

Table 3 gives the thermodynamic values for formation of inhibitory site-niflumic acid complex at 0 °C.

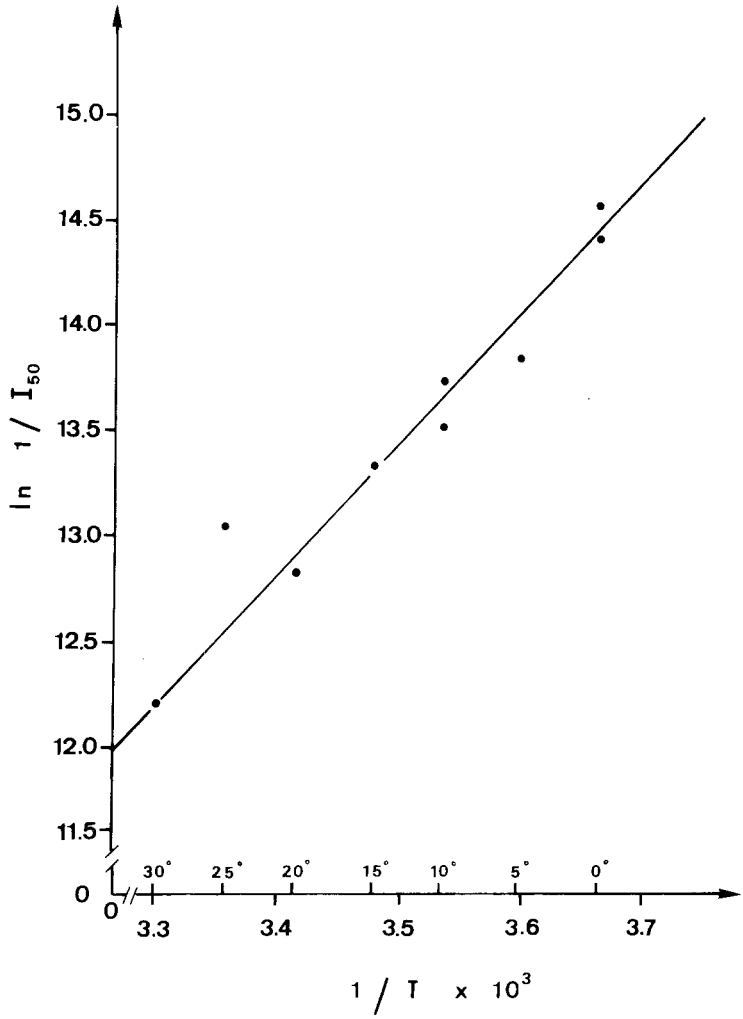


Fig. 11. Inhibition of oxalate self-exchange in human red cell by niflumic acid as a function of temperature. *Ordinate:* natural logarithm of the reciprocal of the niflumic acid concentration causing 50% inhibition of oxalate transport, $\ln(1/I_{50})$. *Abscissa:* reciprocal absolute temperature ($1/T$)

Table 3. Thermodynamic values for formation of inhibitory site-niflumic acid complex at 0 °C

	$+\Delta G$	$-\Delta H$	$-T\Delta S$
	kcal/mol		
Inhibitory site/niflumic acid complex formation	7.8	12.6	4.8

Effect of Niflumic Acid on Other Transport Systems in Red Cells

Glucose self-exchange: We measured the effects of niflumic acid and an analog, meclofenamic acid, on the chloride self-exchange and glucose self-exchange in human red cell. We determined the drug concentrations causing a 50% inhibition of the tracer equilibrium fluxes. As is apparent in Table 4, the glucose exchange is two orders of magnitude less sensitive to these two drugs than the chloride transport. It must be noted that 50% inhibition of glucose self-exchange by meclofenamic acid is ob-

Table 4. Inhibition of chloride and glucose exchange in human red cell (0 °C, pH 7.4) by niflumic acid congeners and phloretin

Compounds	I_{50} (M) glucose self-exchange	I_{50} (M) chloride self-exchange
Niflumic acid	10^{-4}	6.3×10^{-7}
Meclofenamic acid	6.9×10^{-5}	7.5×10^{-7}
Phloretine	1.3×10^{-6}	1.6×10^{-6}

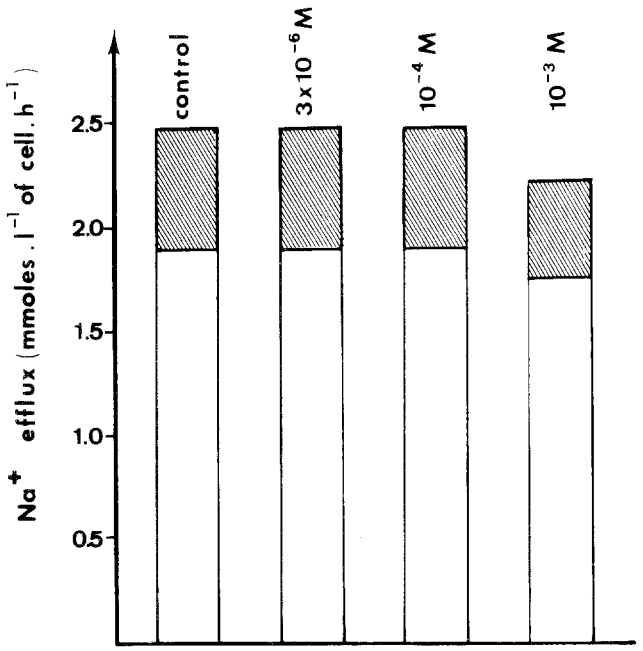


Fig. 12. Effect of increasing niflumic acid concentration on Na^+ efflux in human red cell. ▭ ouabain-sensitive Na^+ efflux; ▨ ouabain-insensitive Na^+ efflux

served at a concentration of this drug which produced 50% of its protective effect against hypotonic hemolysis (Inglot & Wolna, 1968). In contrast, phloretin and niflumic acid methyl ester inhibit the two transport systems to the same extent. The I_{50} values we found for phloretin confirm those reported by Lefevre (1961) for glucose and by Wieth *et al.* (1972) for chloride transport.

Na^+/K^+ active transport and Na^+/Na^+ self-exchange: In human red cells only a fraction of the sodium movement is inhibited by ouabain (Lubowitz & Whittam, 1969; Dunn, 1970). Niflumic acid inhibits the cation movements very poorly. At a concentration as high as 10^{-3} M the ouabain insensitive Na^+ efflux (Fig. 12) is not affected. These results are similar to those obtained by Dunn (1972) with a potent diuretic, triflocin, which is an isomer of niflumic acid.

Discussion

The results reported above show that the anion self-exchange in red blood cells is strongly inhibited by niflumic acid. The inhibitory action is instantaneous and reversible. From the I_{50} value we found, 5×10^{-7} M, this drug is among the most powerful reversible inhibitors of anion transport.

I. Niflumic Acid Interactions with the Protein Involved in Anion Permeability

Analysis of the binding curves of niflumic acid in the presence and in the absence of previous SITS treatment (Fig. 4) suggests that there exist at least two distinguishable populations of niflumic acid binding sites on the erythrocyte membrane:

1) Binding sites which can be protected by SITS and are possibly related to inhibition of anion permeability.

2) Different sites which are not affected by SITS.

Since unsealed ghosts are shown to exhibit more "unspecific" DIDS binding sites than intact cells (Cabantchick *et al.*, 1975), we performed SITS treatment on intact cells prior to the preparation of ghosts. Thus, population 1 represents SITS protectable niflumic acid-binding sites in intact cells. The maximum number of binding sites in population 1 is 0.9×10^6 per cell. This number is in excellent agreement with the number of

band 3 copies per cell [about 10^6 (Fairbanks, Steck & Wallach, 1971; Juliano, 1973)] on which the isothiocyano stilbene disulfonic acids bind with a high degree of specificity. The niflumic acid dissociation constant, K_D, for the binding sites of population 1 is 10^6 M and the drug inhibits the anion self-exchange with a I_{50} value 5×10^{-7} M. The values of K_D and I_{50} are close but not rigorously comparable since K_D refers to a free drug concentration and was measured in unsealed ghosts, whereas I_{50} refers to a total concentration of drug and was measured in intact cells. Furthermore, the concentration of equivalent cells was different in the two cases.

The SITS unprotectable binding sites may involve sites existing in intact cells as well as additional sites which appeared after lysis of cells. Population 2 is not saturable over the concentration range studied. This finding suggests the participation of membrane lipids for this population of binding sites.

On the other hand, the kinetics of the irreversible reaction of SITS with the membrane sites involved in chloride permeability is strongly affected by the presence of niflumic acid in the medium. Thus, it is clear that each inhibitor interferes with the binding of the other.

From the observations discussed above:

1) Niflumic acid inhibits the anion self-exchange at very low concentrations.

2) It inhibits the binding reaction of SITS with anion controlling transport sites.

3) The number of SITS protectable niflumic acid binding sites per cell is equal to the number of band 3 protein copies per cell.

4) Niflumic acid has the same affinity for SITS protectable binding sites as for its anion self-exchange inhibitory sites.

We may conclude with reasonable certainty that niflumic acid interacts with the band 3 protein involved in anion permeability.

II. Features of the Interaction

The curve relating chloride flux to chloride concentration passes through a maximum. This indicates the existence of two counteracting effects. The activating effect is related to the saturation of the transport system with chloride and the inhibitory effect could represent the combination of additional chloride ion with a modifier site (Dalmark, 1976).

Figures 8 and 9 show that the fractional inhibition of chloride fluxes

in red cell ghosts produced by niflumic acid is not affected by increasing the chloride concentration up to 750 mM. Similar results are obtained (not shown) when chloride concentration is varied in intact red cells by nystatin technic (Cass & Dalmark, 1973). This finding indicates that the inhibitor does not compete with chloride either at the transport site or at the modifier site. The niflumic acid is therefore a purely noncompetitive inhibitor.

Niflumic acid has a negatively charged carboxyl group at neutral pH. We measured the effect of altering the pH of the medium on inhibitory action of niflumic acid on chloride self-exchange flux. The I_{50} value was constant between pH 6.0 and 7.2. Since the concentration of the uncharged form varied by a factor of twenty while the negatively charged form was nearly constant, this result indicates that the anionic form of the drug is the active inhibitor. Another argument also supports this conclusion: the inhibitory activity of niflumic acid decreases by a factor of 50 when the carboxylate group is esterified (Table 2). It must be noted that the importance of this observation is emphasized by the following finding. We demonstrated elsewhere, in particular for niflumic acid analogs (J. L. Cousin and R. Motais, *unpublished*), that the hydrophobic character of the molecules was essential in their inhibitory activity and that electrophile substituents increase the inhibitory activity. The hydrophobicity of the drugs was quantified by their $R_{M_{H_2O}}$ value (*see Materials and Methods* and Motais & Cousin, 1976). The greater the hydrophobic nature of these drugs ($R_{M_{H_2O}}$), the greater the inhibitory activity became. The methyl ester niflumic acid derivative was more hydrophobic than niflumic acid ($R_{M_{H_2O}}=3.11$ and 2.45, respectively; Table 2), it is, however, less active. Nevertheless, the unionized form is believed to have a poor inhibitory capacity because the methyl ester derivative of niflumic acid slightly inhibits the chloride flux. The structure activity study quoted above suggests hydrophobic interaction between the drug and its receptor. Typically "hydrophobic interactions" are entropy driven processes which occur with positive ΔS and ΔH decrease with decreasing temperature (being often positive at low temperature). In contrast, Fig. 11 shows that ΔH is constant between 0 and 30 °C, Table 3 shows that both ΔH and ΔS are positive and ΔH is the predominant term. This suggests that Van der Waals-London dispersion forces may contribute an additional driving force in the receptor-inhibitor complex formation. This also suggests that the binding of niflumic acid to its receptor generates some structural changes in the protein and surrounding water in such a way that the overall ΔH and ΔS

changes are negative. In addition, structural modifications induced by niflumic acid in the protein involved in anion transport are also shown by the noncompetitive type of inhibition exhibited by niflumic acid. The drug is proposed to bind to its receptor in two steps: a binding at the protein-water interface to an electrophile group followed by a conformational change in the protein and surrounding water locking the drug in a hydrophobic crevice with electron donor groups; the final result of this process is the alteration of the transport site of the protein.

It is of some interest that DIDS, which presumably inhibits like its analog SITS, acts as a competitive inhibitor of chloride permeability at the transport site (Shami, Rothstein & Knauf, 1978), and it seems reasonable to assume that irreversible binding occurs at the reversible binding site according to the equation:

$$I + T \underset{\longleftarrow}{\overset{K_i}{\longrightarrow}} \underset{\text{reversible}}{IT} \xrightarrow{k} \underset{\text{irreversible}}{IT}$$

where I is the inhibitor concentration, T the transport site concentration, K_i the dissociation constant of the reversible complex IT, k the rate constant of the irreversible reaction.

Niflumic acid interferes with irreversible SITS binding and reciprocally. This finding could indicate that:

1) binding of niflumic acid to the protein induces a conformational change of the anion transport site and thus decreases the rate of the irreversible SITS binding. However, from our data it is not possible to distinguish between an effect on the affinity of reversible SITS for the transport site (K_i increased) or an effect on the rate of the irreversible reaction (k decreased) or both effects simultaneously.

2) Irreversibly bound SITS modifies the conformation of the niflumic acid site in such a way that drug binding becomes impossible.

III. Specificity of the Drug

The data given are strongly in favor of a direct binding of niflumic acid to the band 3 protein. This band does not seem homogeneous. Proteins that migrate at the same rate in polyacrylamide gel electrophoresis mediate the transport of water (Pinto da Silva, 1973; Brown, Feinstein & Sha'afi, 1975), possibly the cation movement (Avruch & Fairbanks, 1972), and sugar [(Taverna & Langdon, 1973; Lin & Spudich, 1975) but recently, sugar transport protein has been identified as band 4.5

(Kasahara & Hinkle, 1977)]. For this reason we tested the effect of niflumic acid on glucose and cation movement across the human red cell membrane.

The present results show that niflumic acid is much more active against anion exchange than against glucose and cation transport systems in human red cells. This drug is therefore a relatively specific inhibitor of anion permeability.

Inhibition of Glucose Transport

Glucose transport across human red cell is two orders of magnitude less susceptible to niflumic acid and meclofenamic acid than anion exchange (Table 4). Meclofenamic acid (a structural analog of niflumic acid) has been tested against hypotonic lysis of human red cells (Inglot & Wolna, 1968). It inhibited glucose transport by 50% at a concentration at which 50% of its protective effect against hemolysis is observed. Hemolysis protection has been related to the expansion of the membrane due to the presence of the drugs within membrane lipids (Seeman, 1972). Thus, the inhibitory effect of niflumic acid and meclofenamic acid on glucose transport could result from their adsorption to the membrane lipids, leading to an expansion of the bilayer and a structural change in the translocator. However, further investigations are necessary to determine whether these drugs affect glucose transport by expansion of the lipid bilayers or by direct interaction between the drugs and the protein involved in the transport.

Inhibition of Cation Transport

To interpret the effects of anionic drugs on anion permeability of the membranes, it has been proposed that the hydrophobic adsorption of the compounds to the lipids produce a negative potential at the membrane-water interface (Fortes & Hoffman, 1974; McLaughlin, 1973). Such a negative surface potential would decrease the anion concentration at the membrane surface and consequently the transmembrane anion fluxes. This hypothesis predicts a stimulation of the cation fluxes in the presence of these drugs. However, the present results show that no increase in cation transport is observed at a niflumic acid concentration ($\simeq 10^{-6}$ M) that inhibits anion exchange substantially, and furthermore that cation

transport is inhibited by high drug concentrations (10^{-3} M). Thus, a purely electrostatic mechanism is difficult to reconcile with the observed effects of niflumic acid on cation transport of human red blood cell membranes.

Other anionic amphiphilic compounds, including ANS, ethacrynic acid and furosemide, also inhibit anion exchange in human and bovine red cell at low concentrations and Na^+ ouabain insensitive fluxes at high concentrations (Hoffman & Kregenow, 1966; Lubowitz & Whittam, 1969; Deuticke, 1970; Dunn, 1970; Sachs, 1971; Motais & Sola, 1973; Cousin & Motais, 1976; Motais & Cousin, 1976*a*, *b*; Brazy & Gunn, 1976). These drugs induce a disc-echinocyte transformation of the red cell (Deuticke, 1970; Fortes & Ellory, 1975). From the similarity between the dose-response curves for inhibition of cation transport, shape changes of the cell, and hemolysis protection, Fortes and Ellory (1975) suggested that inhibition of cation transport by ANS is a consequence of an expansion of bilayer due to the interaction of the drug with the membrane lipids. Niflumic acid has not so far been quantitatively tested on cell shape change and against hypotonic hemolysis, but structural analogs exhibit their protective effect against hemolysis at 10^{-3} M (Inglot & Wolna, 1968), a concentration at which niflumic acid inhibited Na^+ ouabain-insensitive flux. It is therefore possible that the inhibition of cation transport results from an expansion of the bilayer.

In conclusion, the inhibition of anion self exchange in human red cell by niflumic acid results from a direct drug-protein interaction, whereas the inhibition of glucose and cation transport could be due to an expansion of the bilayer resulting from an absorption of the drug to the lipids.

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