J. Membrane Biol. 64, 23–31 (1982)

Effects of the Plant Alkaloid Sanguinarine on Cation Transport by Human Red Blood Cells and Lipid Bilayer Membranes

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Summary. The plant alkaloid, sanguinarine, inhibits the ouabain-sensitive K – Na pump and increases the downhill, ouabain-insensitive movements of K and Na in human red cells. These two effects have different temporal and concentration dependencies and are mediated by two different chemical forms of sanguinarine. The oxidized, charged form (5×10^{-5} M) promptly inhibits the pump but does not affect leakage of K and Na. The reduced, uncharged form of sanguinarine causes lysis of red cells but does not inhibited the pump. Sanguinarine also increases the conductance of bilayers formed from sheep red cell lipids. The effect is produced by the uncharged but not by the charged form of sanguinarine. Bilayer conductance increases as the fourth power of sanguinarine concentration when the compound is present on both sides of the membrane and as the second power of concentration when present on only one side. Conductance also increase *e*-fold for each 34 mV increase in the potential difference imposed across the membrane. The results suggest that the uncharged forms of sanguinarine produce voltage-dependent channels in bilayers.

Key words sanguinarine · cation transport · red cells · lipid bilayer membranes

Introduction

Sanguinarine (pseudochelerythrine) belongs to the benzophenanthridine alkaloids (Merck Index, 1976; Manske, 1954). This class of compounds has been shown to inhibit yeast respiration (Vallejos & Roveri, 1972), to abolish oxidative phosphorylation in rat liver mitochondria (Vallejos & Rizotto, 1972), to uncouple photophosphorylation in spinach chloroplasts (Vallejos, 1973), and recently Straub and Carver (1975) have demonstrated that sanguinarine is also an inhibitor of the Na^+, K^+-dependent ATPase prepared from guinea pig brain. The results of studies performed on frog skeletal muscle show that sanguinarine increases the cation conductance as well as inhibiting the Na, K-ATPase of that preparation (Chan & Moore, 1976). In addition, sanguinarine affects the cation permeability barriers at the outer surface of the frog skin (Nichols, Straub & Abermathy, 1978).

The present report describes experiments showing that sanguinarine at relatively low concentration (20–30 μm) completely inhibits the sodium pump of erythrocytes without affecting the leak for K^+ and Na^+. At concentrations higher than those necessary for pump inhibition, sanguinarine markedly increases the permeability of the erythrocyte membrane to K^+ and Na^+. We also report that sanguinarine increases the conductance of thin lipid membranes. The sanguinarine-induced conductance in bilayers exhibits a high power dependence upon sanguinarine concentration. We present evidence that the charged form of the compound is responsible for pump inhibition while the uncharged form produces increased cation permeability in both red cells and bilayers.

Materials and Methods

All glassware was washed with concentrated nitric acid (rubber stoppers and the like with dilute nitric acid) and distilled water before use. Irreproducible inhibition of pump fluxes by some unknown substance(s) was observed if this washing procedure was omitted. All reagents were analytical grade.

Red Blood Cells

Venous blood (always from J.G.N.) was sampled in heparinized tubes, centrifuged at $28{,}000 \times g$ for 10 min, and plasma and white cells were aspirated and discarded. The RBCs were resuspended in about 3 volumes 165 mM KCl, and the centrifugation was repeated. The cells were then washed twice (centrifuge force up to $17{,}000 \times g$ and down) in the cold with the flux-medium (*see* below). After the final centrifugation enough medium was left to give a cell suspension of about 50% hematocrit. In some cases separate samples of cells, plasma, or medium were isolated by centrifugation in small nylon tubes (Funder & Wieth, 1966*a*; Gunn, Dalmark, Tosteson & Wieth, 1973) and analyzed (*see* below). In all cases cells were prepared immediately before use.

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0022-2631/82/0064-0023 $01.80

Flux-Medium

The composition of the flux-medium was (conc. in mM): Na^+, 140; K^+, 5; Cl^-, 139; glycylglycine$^-$, 6; glycylglycine, 21; glucose, 5; in distilled water. The measured pH (38 °C) and osmolarity of this medium were 7.4 and 295 mOsm, respectively. When appropriate, 200 µl 10^{-2} M ouabain in the medium were added per 20 ml medium (final conc. 10^{-4} M). Stock solutions of sanguinarine up to about 1.3×10^{-2} M were made by dissolving sanguinarine sulphate (Pfalz and Bauer, Flushing, N.Y. 11368, used without further purification) in dimethylsulfoxide (DMSO), and in all experiments 200 µl DMSO with or without sanguinarine were added per 40 ml medium unless otherwise indicated.

$^{42}K_2CO_3$ was purchased from North Carolina State University, Raleigh, N.C., and dissolved in HCl to yield a 160-mM, neutral solution of KCl with 5–15 Ci/mol K^+ at the time of the experiment.

Flux Experiments

In a typical experiment a number of 50-ml, rubber-stopped Erlenmeyer flasks were gently shaken on a water bath at 37 °C. Each flask contained 40 ml medium ± ouabain 10^{-4} M, washed RBCs to a final hematocrit of 3% (unless otherwise stated), 200 µl DMSO ± sanguinarine and 50–100 µl ^{42}K-solution (*see* above). The red cells were added at a hematocrit of 50% (cold suspension) to the prewarmed medium, and after 5 min the tracer flux experiment was initiated by addition of $^{42}K^+$. Sampling consisted of removal of 3- to 5-ml aliquots from all flasks at predetermined interval during the 2–2½ hr flux period. The samples were added to prechilled, isotonic $MgCl_2$ in 12 ml polycarbonate centrifuge tubes and the cells washed as described by Hoffman and Tosteson (1971). After three washings the cells were lyzed in the tubes by addition of an appropriate amount (usually 3 ml) of hypotonic CsCl (10 mM) solution containing a small amount of detergent. The lysate was used for determination of radioactivity, K^+, Na^+, and hemoglobin as described below. The water content of cells and the composition of the media were determined on separate samples by centrifugation in small nylon tubes (Funder & Wieth, 1966*a*; Gunn et al., 1973) at the beginning and the end of the experiment.

Analytical Methods

Radioactivity in the cell lysates and media samples was counted in an intertechnique CG 30, Gamma Scintillation Counter (Intertechnique Ltd., Fairfield, N.J.), the polycarbonate tubes being placed in special adaptors in the counter.

K^+ and Na^+ were determined by atomic absorption flame photometry (Perkin Elmer, 303). The standards of KCl and NaCl were made in the hypotonic CsCl solution which was also used for hemolysis of the cell samples. All analyses were performed in CsCl in order to minimize interference between Na^+ and K^+ (Funder & Wieth, 1966*a*).

Hemoglobin concentration was determined by measuring optical density at 540 nm, OD_{540} (Gilford 300-N spectrophotometer) of a mixture of 0.25 ml cell lysate and 2 ml Drabkin-solution (280 mg KH_2PO_4, 400 mg $K_3Fe(CN)_6$, 100 mg KCN and 1 ml non-ionic detergent in 1 liter H_2O (stored in a black bottle)). The concentration (g/liter) was equal to $OD_{540} \times 1.465$.

Water content of RBC was obtained by drying a weighed cell sample at 95–100 °C for 24 hr (Funder & Wieth, 1966*a*).

Microhematocrits were determined by means of a microcapillary centrifuge (International Equipment Co., Needham Heights, Mass.) and an optical comparator designed by T.J. McManus (Department of Physiology, Duke University, Durham, N.C.).

Table 1. Definition of symbols

Subscripts: i = inside (cells), o = outside
1 = sample number 1, 2 = sample number 2

$^{42}K_i$ = radioactivity, cpm · (kg hemoglobin)$^{-1}$
K_i = meq K^+ · (kg hemoglobin)$^{-1}$
a = specific activity, cpm · (meq K^+)$^{-1}$ = $^{42}K/K$
t = time
M_i = influx, meq K^+ · (kg hemoglobin)$^{-1}$ · hr^{-1}
M_o = efflux, meq K^+ · (kg hemoglobin)$^{-1}$ · hr^{-1}

Calculations

K^+ influx by tracer experiments: The symbols used are given in Table 1. Briefly, samples for determination of $^{42}K_i$, K_i and a_o are taken at different points during an experiment. A "flux period" is here defined as the period between two samplings. The net influxes of K^+ and ^{42}K are given by

$$\frac{dK_i}{dt} = M_i - M_0 \quad \text{and} \quad \frac{d^{42}K_i}{dt} = M_i \cdot a_o - M_o \cdot a_i.$$

Inspection of the experiments (in which the net flux of K^+ is small) shows that the following equations hold for the flux periods:

$$\frac{dK_i}{dt} = \frac{(K_i)_2 - (K_i)_1}{t_2 - t_1} = k'$$

$$\frac{d^{42}K_i}{dt} = \frac{(^{42}K_i)_2 - (^{42}K_i)_1}{t_2 - t_1} = k''$$

$$a_o(\text{in a given flux period}) = \frac{(a_o)_1 + (a_o)_2}{2}$$

(a_o is nearly constant)

$$a_i(\text{in a given flux period}) = \frac{(a_i)_1 + (a_i)_2}{2}$$

(a_i increases linearly with time; it is small compared with a_o).

Combination of these equations gives:

$$k'' = M_i \cdot a_o - (M_i - k') \cdot a_i$$

from which

$$M_i = \frac{k'' - k' \cdot a_i}{a_o - a_i}$$

where k'', k', a_i and a_o can all be determined experimentally for each flux period. "Pump flux" was calculated as M_i in the absence of ouabain minus M_i in the presence of this inhibitor (leak flux).

Net fluxes of K^+ and Na^+ were calculated from measurements of K^+ and Na^+ in the cells (meq · (kg hemoglobin)$^{-1}$) as a function of time during the first hour of the experiments. This procedure also allowed the determination of cell composition at time zero (Table 2, footnote c), either by direct measurement or by extrapolation of the apparently linear relationship between cell concentrations of K^+ or Na^+ and time.

Characteristics of the RBC's Used. Accuracy and Reproducibility of the Experiments

Table 2 summarizes a number of measurements on the red cells used in the present experiments over a period of about six months.

Table 2. Characteristics of red blood cells used in the present experiments

Substance	Units	Mean	SD	SEM	*n*
Composition of RBC[a] (concentrations)					
Cell solids	g·(kg cells)$^{-1}$	350	3	1	9
H_2O	g·(kg cell solids)$^{-1}$	1,860	28	9	9
Hemoglobin (Hgb)	g·(kg cell solids)$^{-1}$	864	18	6	9
K^+	meq·(kg Hgb)$^{-1}$	294	9	3	7
Na^+	meq·(kg Hgb)$^{-1}$	22.4[b]	1.4	0.6	6
Fluxes[c] (meq·(kg Hgb)$^{-1}$·hr^{-1}), influx positive					
K^+ net flux	No ouabain	−3.1	3.6	0.9	18
	10^{-4} M ouabain	−2.1	4.0	1.0	16
Na^+ net flux	No ouabain	−1.8	1.1	0.3	17
	10^{-4} M ouabain	2.9	1.1	0.3	16
K^+ influx (tracer measurements)					
Total		5.63	0.12	0.03	13
Ouabain sensitive		4.23	0.14	0.04	13
Ouabain insensitive		1.41	0.10	0.03	14

[a] Red blood cells isolated from venous blood by centrifugation in small nylon tubes (*see* Methods).
[b] Corrected for 3% plasma contaminating the packed erythrocytes (Funder & Wieth, 1966*a*, *b*).
[c] Control experiments (without sanguinarine) on washed cells as described under Methods. The composition of these washed cells in meq·(kg Hgb)$^{-1}$ were (mean ± SD): $K^+ = 284 \pm 12$ ($n = 27$); $Na^+ = 22.4 \pm 2.0$ ($n = 29$).

The cell composition shows minor variability and falls within the normal range for human erythrocytes (e.g., Funder & Wieth, 1966*b*). The net fluxes of Na^+ and K^+ are small and insignificant except in the case of the leak net influx of Na^+. The tracer influx values for K^+ compare well with those given by Funder and Wieth (1967) for comparable experimental conditions. The accuracy and reproducibility of the measurements seem satisfactory.

Lipid Bilayers

The lipids used in the presented study were obtained from high potassium sheep red blood cells following the extraction procedure described by Andreoli, Bangham and Tosteson (1967*a*). Thus, the final extract yielded a phospholipid cholesterol mixture with a molar ratio of 1:1 (Andreoli & Monahan, 1968). The extract was dissolved in *n*-decane such that there was approximately 20 mg lipid/ml solution. The membranes were formed across a polyethylene partition with a smooth hole of 1.8×10^{-2} cm^2 area by the pipette technique of Szabo, Eisenman and Ciani (1969). The partition over which the membranes were formed separated two chambers, 1.5 ml each. The chambers were ported such that during the course of an experiment the solutions could be changed by means of matched syringes. The volumes of the chambers, and therefore membrane surface area, were kept constant by addition or removal of fluid with a Gilmont syringe (Great Neck, N.Y.). The solutions used to bathe the bilayers were buffered at the appropriate pH with 3 mM phosphate buffer. All measurements to be reported were made upon membranes which were optically black at 23 °C. Addition of substances to the bathing media was accomplished directly with a Hamilton syringe (Reno, Nev.) or by perfusion with the appropriate media. The individual chambers were well stirred with magnetic fleas. Measurements of membrane potential (V_m) and current (I_m) were made using a previously described four-electrode clamp apparatus (Andreoli & Troutman, 1971).

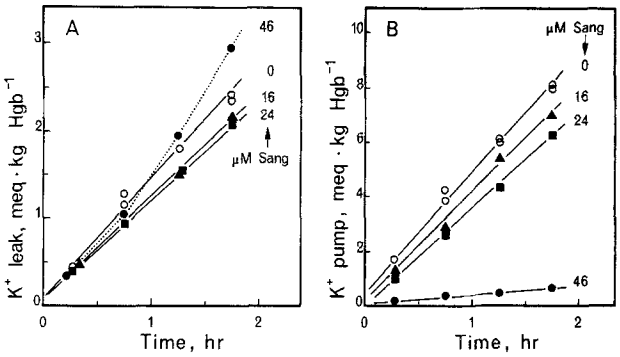


Fig. 1. The effect of various concentrations of sanguinarine upon (*A*) unidirectional leak influx of K^+ as a function of time, and (*B*) the oubain-sensitive K^+ influx (pump) measured using human red blood cells as described under Methods. The hematocrit was 3%

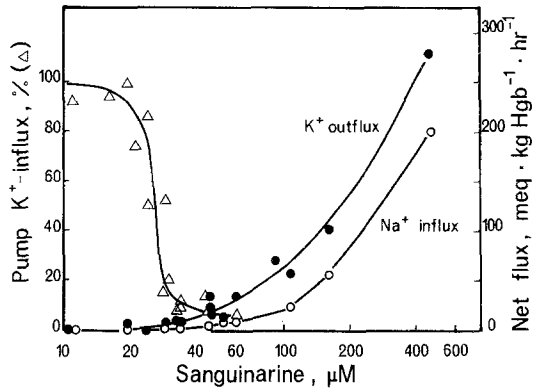


Fig. 2. The effect of sanguinarine upon pump and leak fluxes in human red blood cells (hematocrit = 3%). The pump K^+ influx (△) is expressed as % of control, while net K^+ efflux (●) and Na^+ influx (○) are expressed in meq × kg hemoglobin^{-1} × hr^{-1}. The net fluxes are taken as the average flux in the period 0.5 to 1.5 hr after the start of the experiment

Results

The Effect of Sanguinarine on Pump and Leak Fluxes

Typical results revealing the effect of sanguinarine on leak and pump influx of K^+ are shown in Fig. 1. It appears that in concentrations up to about 24 μM, sanguinarine affects neither leak nor pump flux. At 46 μM sanguinarine the pump is nearly 100% inhibited. The leak, however, is only affected at this concentration after more than one hour of incubation (Fig. 1*A*). As shown below, the effect on the pump flux is probably due to the oxidized, cationic form of sanguinarine.

Figure 2 gives the observed relationship between sanguinarine concentration and pump and leak fluxes.

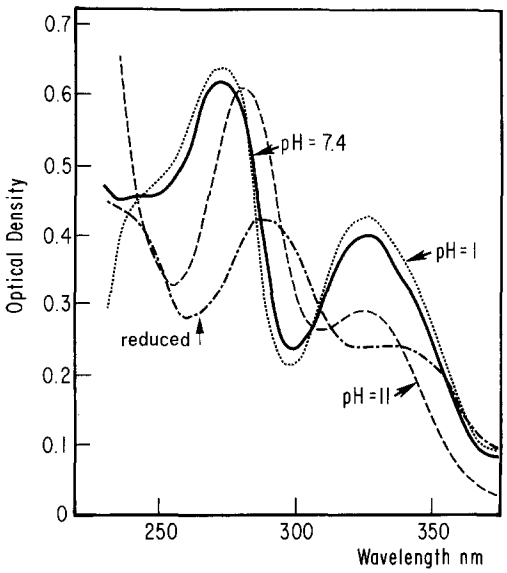


Fig. 3. Absorption spectra (1 cm cuvette) of 22.5 μM sanguinarine solutions at different pH. 100 μl DMSO with sanguinarine was added to 20 ml flux medium (pH 7.4), and pH was changed by addition of 60 μl 1 N HCl or 60 μl 1 N NaOH. The spectrum marked "reduced" is recorded after addition of 60 μl 50 mM DTT to the solution represented by the pH=7.4 spectrum. 20 ml medium + 100 μl DMSO was used as the blank

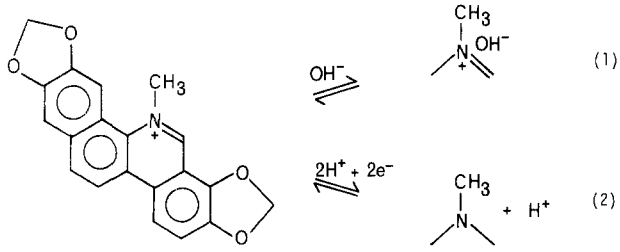
It must be emphasized that on the abscissa (logarithmic scale) is shown the concentration of sanguinarine which would obtain if there was zero uptake by the red blood cells. Judging from Fig. 6 (*see* below), the "free" concentration of sanguinarine may well be only 10% of the "total."

Figure 2 clearly shows that, in addition to the inhibitory effect of sanguinarine on the pump, there are marked increases in dissipative Na and K fluxes at sanguinarine concentrations in excess of those necessary for complete pump inhibition. This may be fortuitous or may suggest that sanguinarine acts on some part of the membrane structure which is important for both the active and passive properties of the red cell membrane. The rate of K^+ outflux seem to be consistently higher than that of Na^+ influx, probably reflecting a difference in permeability of the two ions (the electrochemical potential differences ($\Delta\tilde{\mu}$) are approximately equal: $\Delta\tilde{\mu}_K = -75$ mV, while $\Delta\tilde{\mu}_{Na} = +72$ mV ($V_m = -10$ mV).

The "concentration" of sanguinarine producing 50% inhibition of the pump is around 25 μM (probably corresponding to a concentration of about 2 μM in the medium). These values are close to the I_{50} = 10 μM for the inhibition of yeast respiration (Vallejos & Roveri, 1972) by sanguinarine and the concentration required for half maximum inhibition of guinea pig brain Na, K-ATPase, 5 μM (Straub & Carver, 1975). The inhibition curve in Fig. 2 is much steeper than would be expected if the inhibition was simply the result of a 1-to-1 reaction of a pump molecule with a sanguinarine molecule (*compare*, for instance, with Fig. 1 of Vallejos & Roveri, 1972). The apparent high degree of "positive cooperativity" in the inhibition is, however, difficult to interpret in molecular terms since the true concentration of sanguinarin is unknown.

Identification of the Chemical Form of Sanguinarine that Inhibits the Pump

Sanguinarine has both acid-base, and redox properties linked to its principal functional group:



From Fig. 3, which gives the absorption spectra of sanguinarine at different pH values, it may be deduced that the pK of sanguinarine is higher than 7.4 (calculations based on the difference between the spectra at pH=1 and pH=7.4 and assuming full titration at pH=11 gives a pK of about 8.5) and that the oxidized, cationic form is predominant under the conditions of the flux experiments. The cationic form is yellow-orange in solution, whereas the neutral form is only slightly yellow.

At neutral or alkaline pH (corresponding to the pH=7.4 or pH=11 spectra in Fig. 3) addition of the reducing agent dithiothreitol (DTT, Clelands reagent) causes the large absorption peak to shift to higher wavelengths and both peaks diminish in intensity corresponding to a decolorization of the solution (Fig. 3). The effect of DTT can be reversed by $Fe(CN)_6^{3-}$ or by addition of H^+ as expected from Eq. (2). The reduced form of sanguinarine, dihydrosanguinarine, is less soluble than the oxidized form and the solutions tend to become hazy.

The effects of DTT and $Fe(CN)_6^{3-}$ upon red blood cells bathed in sanguinarine-containing media are shown in Fig. 4. These data show that it is the oxidized form of sanguinarine that is responsible for inhibition of the pump since the inhibition is the same with and without $Fe(CN)_6^{3-}$. Figure 4 also shows by comparison with Fig. 1*A* that the cation leak is not increased under these conditions. In contrast, reduction of sanguinarine with DTT produces immediate and complete hemolysis of red cells, making quan-

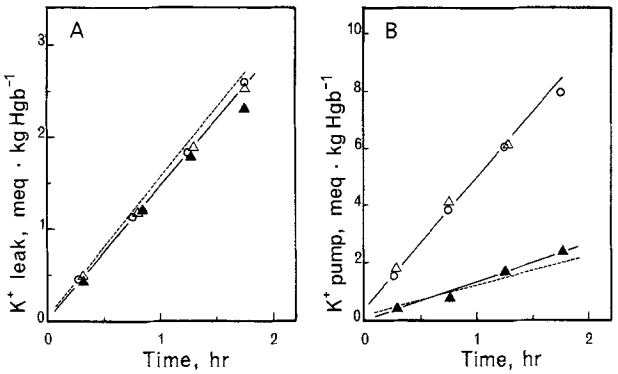


Fig. 4. Effect of 0.1 mM dithiothreitol (DTT), (○), or 1.7 mM $K_3Fe(CN)_6$, (△, ▲) on (*A*) leak and (*B*) pump fluxes in the absence (○, △) or presence (▲) of 35 μM sanguinarine. Hematocrit was 3.2%. In the ferricyanide experiments, $K_3Fe(CN)_6$ was substituted for KCl in the medium to give 5 mM K^+ (*see* "flux-medium", Methods). In the presence of DTT *and* sanguinarine the medium became hazy and even the first samples showed pronounced hemolysis. The broken line is an experiment with 35 μM sanguinarine without DTT and $Fe(CN)_6^{3-}$

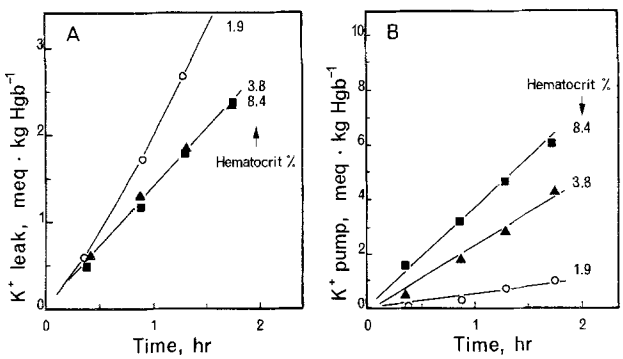


Fig. 5. The effect of hematocrit on pump and leak at constant sanguinarine concentration. The same type of experiment as in Fig. 1 except for the fact that the hematocrit was *varied* as shown and calculated sanguinarine concentration was *constant* 37 μM

titative experiments impossible. That the above are induced effects of the oxidized and reduced forms of sanguinarine is illustrated by Figs. 4*A* and *B*, where it is shown that $Fe(CN)_6^{3-}$ and DTT are ineffective in altering cation transport by red cells when applied in the absence of sanguinarine.

In analogy with the above observations, K.D. Straub and P. Carver (*personal communication*) report that bathing of frog skin in sanguinarine-containing solutions results in decolorization of the solution, probably due to uptake of sanguinarine by the skin, since this is not seen in experiments with isolated ATPase (Straub & Carver, 1975). They have also shown (through personal communication with Straub and Carver) that dihydrosanguinarine is not an inhibitor of Na, K-ATPase from guinea pig kidney and that the inhibition by sanguinarine can be reversed

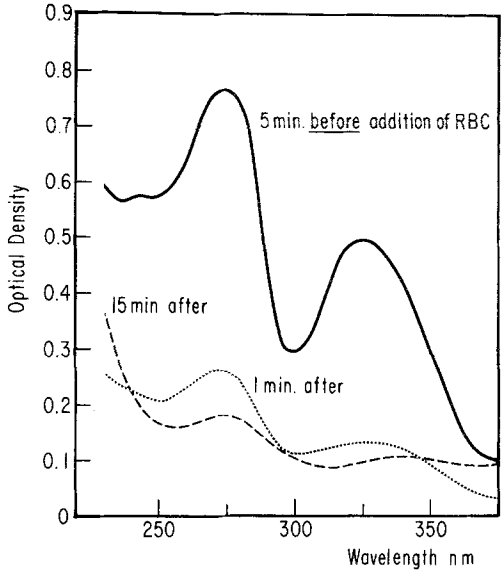


Fig. 6. Spectra of sanguinarine-containing (30 μM) flux-media before and after addition of erythrocytes. Hematocrit = 4%

by reducing agents, supporting the idea that the form which is inhibitory to the pump is the oxidized form of sanguinarine.

Partition of Sanguinarine between the Red Blood Cells and the Medium

During the preliminary phase of this investigation we found it difficult to reproduce the results on pump inhibition from experiment to experiment and it was observed that relatively small variations in hematocrit were the principal cause of irreproducibility. This is illustrated by Fig. 5. At the constant, calculated, sanguinarine concentration of 37 μM there is very little inhibition when the hematocrit is 8.4% but almost complete inhibition in the 1.9% hematocrit experiment, in which the leak is also slightly affected. An observation relevant to this point is the following. When red blood cells are added to the yellow-orange, sanguinarine containing flux-medium the medium is decolorized as illustrated by the spectra in Fig. 6. Apparently, most of the sanguinarine is removed from the medium by the red cells since the intensity (rather than the position) of the peaks is drastically diminished. Similar spectra (not shown) from experiments with constant, calculated sanguinarine concentration but varying hematocrit clearly show that the concentration of "free" sanguinarine in the medium decreases with increasing hematocrit.

Lipid Bilayer Experiments

In order to explore further the mechanism by which sanguinarine induces an increased cation leak in the

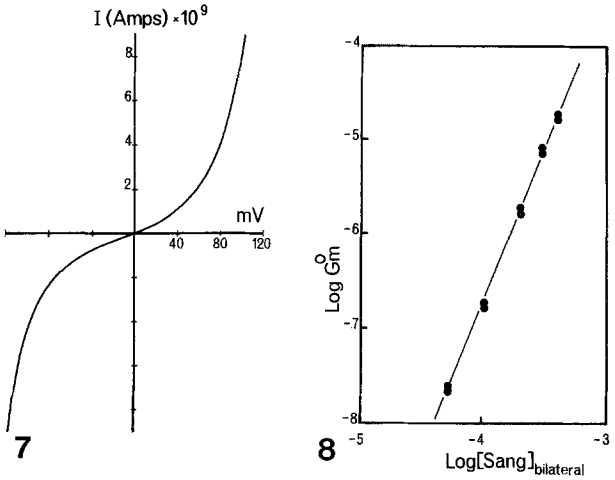


Fig. 7. Current/voltage relationship of a lipid bilayer membrane symmetrically bathed in 0.1 M NaCl and 2×10^{-4} M sanguinarine at pH=7.4. The membrane was formed of sheep RBC lipid in *n*-decane and allowed to become optically black prior to addition of sanguinarine. This and all subsequent measurements were obtained following the stabilization of G_m^o (20–30 min following BLM exposure to sanguinarine)

Fig. 8. The effect of 5×10^{-5} to 4×10^{-4} M sanguinarine upon the membrane conductance measured at the limit of zero current and voltage (G_m^o in $\Omega^{-1}\cdot cm^{-2}$) of lipid bilayer membranes symmetrically exposed to sanguinarine and 0.1 M NaCl at pH=7.4. Slope=3.7, $r>0.09$

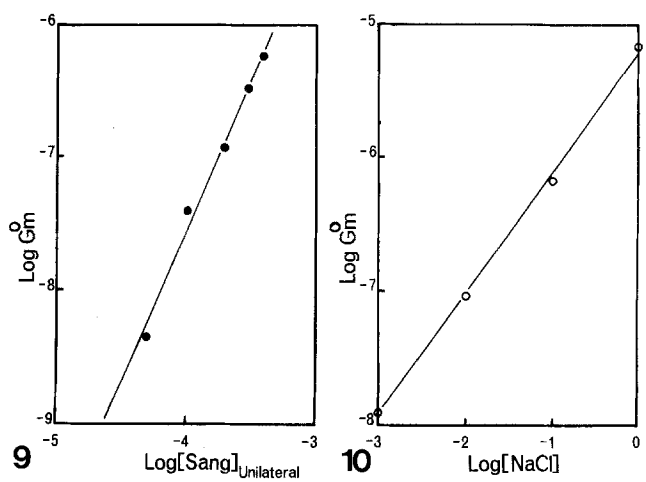


Fig. 9. Membrane G_m^o ($\Omega^{-1}\cdot cm^{-2}$) as a function of unilateral sanguinarine 5×10^{-5} to 4×10^{-4} M at pH=7.4. NaCl was 0.1 M on both sides of the membrane. Slope=2.1, $r>0.99$

Fig. 10. Membrane G_m^o ($\Omega^{-1}\cdot cm^{-2}$) as a function of [NaCl], M, which is the same on both sides of the membrane. Bilateral sanguinarine (2×10^{-4} M) pH=7.4. Slope=0.9, $r>0.99$. While the absolute magnitude of G_m^o varied the slope of the line relating G_m^o to [NaCl] was unaffected if measurements were made on membranes which had been exposed to 2×10^{-4} M sanguinarine yet were bathed in sanguinarine-free medium

red cell membrane, we studied its effect on the electrical properties of thin bilayers formed from lipids extracted from red cell membranes. These and all subsequent data were obtained at steady state, that is when the membrane conductance (G_m^o) became invariant with time after the addition of sanguinarine. This usually took 20–30 min regardless of the bulk sanguinarine concentration. Figure 7 is plot of current (I) as a function of voltage (V) imposed across a membrane exposed to aqueous solutions containing 2×10^{-4} M sanguinarine. The membrane conductance at the limit of zero current and voltage (G_m^o) was about 7×10^{-7} Ω^{-1} cm^{-2} as compared to an average value for bilayer conductance of $3.6\pm0.5\times10^{-9}$ Ω^{-1} cm^{-2} in the absence of sanguinarine. Thus at 0.1 M NaCl and pH 7.4, 2×10^{-4} M sanguinarine raises the G_m^o of lipid bilayers by more than two orders of magnitude. This is consistent with the red cell data presented in Fig. 2 which shows that sanguinarine at this concentration (2×10^{-4} M) increased net fluxes of K^+ and Na^+ by about two orders of magnitude. In light of these observations, it is reasonable to conclude that sanguinarine affects cation permeability in both lipid bilayers and red cell membranes by perturbing the arrangement of lipid molecules.

To explore further the interaction of sanguinarine with lipid bilayers, we measured the conductance as a function of the sanguinarine concentration applied symmetrically to bilayers in 0.1 M NaCl (Fig. 8). These data shown that there is a 4th power dependence of G_m^o on the sanguinarine concentration. In contrast, if asymmetrically applied, the membrane conductance depends upon the second power of the sanguinarine concentration (Fig. 9). Figure 10 shows the sanguinarine-dependent conductance of bilayers plotted as a function of the salt (NaCl) concentration in the solutions bathing the bilayer. The slope of the line is 0.9 which is not significantly different from unity. The steady-state conductance of bilayers exposed to sanguinarine on both sides can be summarized by the expression:

$$G_m^{Sang}=K[NaCl][Sang]^4\exp(0.7\,eV/kT)$$

where K has the value of $7.54\,\Omega^{-1}\cdot cm^{-2}\cdot M^{-5}$.

The large dissipative fluxes of both Na and K observed in sanguinarine-treated red blood cells raises the question of selectivity. In order to evaluate the selectivity of the sanguinarine-induced conductance, experiments were performed in asymmetric salt. In the process of performing these experiments, we found that removal of all sanguinarine from the bulk solution (subsequent to stabilization of G_m^o) resulted in a G_m^o decrease of less than 20% which occurred

Table 3. V_m as a function of asymmetric salt bathing bilayer membranes which had been exposed bilaterally to sanguinarine (4×10^{-4} M)

Salt		V_m (mV)		$G_m^o\times10^a$ (Ω^{-1} cm^{-2})	
Front chamber	Rear chamber				
0.1 M NaCl	0.1 M NaCl	0	(12)	1.50±0.2	(12)[b]
0.1 M NaCl	0.01 M NaCl	+31	±0.92 (11)	0.93±0.03	(11)
0.1 M NaCl	1.0 M NaCl	−30	±2.1 (9)	6.95±1.4	(9)
0.1 M NaCl	0.1 M KCl	−16.5	±2.7 (4)	5.80±1.1	(4)

[a] The steady-state conductance at the limit of zero current and voltage.
[b] Mean ± SEM (n).

immediately and remained stable. Consequently the experiments listed in Table 3 were performed with sanguinarine-treated membranes in sanguinarine-free medium. Since for the single salt case

$$E_{Na} = -E_{Cl}$$

at the limit of zero current and assuming all other charge carriers are negligible with respect to Na and Cl, we can write:

$$V_m = E_{Cl}(2t_{Cl}-1).$$

Inserting the values from Table 3 we calculate

$$t_{Cl}=0.23 \text{ and } t_{Na}=0.77.$$

Thus the sanguinarine-induced conductance appears to be moderately cation selective. The last row in Table 3 depicts the biionic case of NaCl and KCl. The negative potential on the side containing KCl illustrates that the sanguinarine-induced conductance pathway is selective for K over Na, in agreement with observations made in the red blood cell studies.

In order to identify the form of sanguinarine that increases G_m^o, we performed experiments in the presence of oxidizing and reducing agents as well as experiments in media at different pH. Addition of sanguinarine (4×10^{-4} M) to bilayers bathed in media containing excess oxidizing agent, $Fe(CN)_6^{3-}$, produced G_m^o increases which were only 3% of those observed in the absence of $Fe(CN)_6^{3-}$ and sanguinarine. Thus the oxidized form of sanguinarine is only minimally effective in altering G_m^o. In contrast, as was the case with red blood cells, the addition of excess reducing agent (DTT) to media containing sanguinarine resulted in immediate breakage of the lipid bilayer membranes.

The DTT-induced hemoysis of red blood cells and disruption of lipid bilayers in sanguinarine-containing media can be understood as follows. We propose that

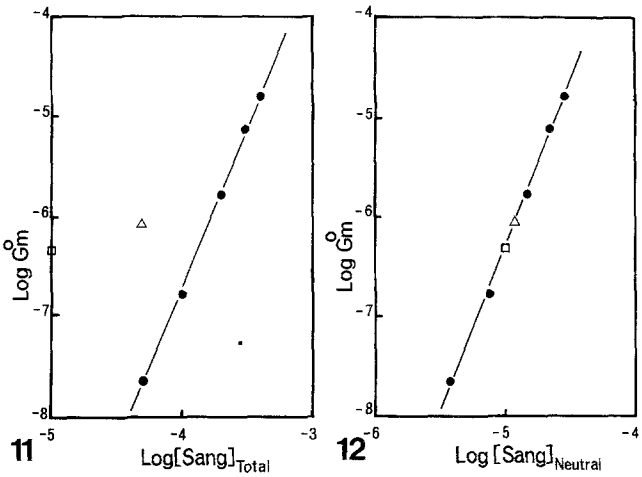


Fig. 11. G_m^o ($\Omega^{-1}\cdot$cm^{-2}) as a function sanguinarine concentration over a range of 5×10^{-5} to 4×10^{-4} M total sanguinarine. Measurements were made at pH=7.4 (●) (data from Fig. 8), 8 (△) and 10 (□). At pH=6 sanguinarine had no effect upon G_m^o, and the points were therefore not included in the graph

Fig. 12. G_m^o ($\Omega^{-1}\cdot$cm^{-2}) as a function of the concentration of the neutral form of sanguinarine. Calculations were made using the Henderson-Hasselbach equation and a pK value of 8.5. The graph includes points obtained at total sanguinarine concentrations ranging from 1×10^{-5} to 4×10^{-4} M (Fig. 11). Measurements were made at pH=7.4 (●), 8(△) and 10 (□). Slope=3.65, $r>0.99$

it is the uncharged form of sanguinarine which is responsible for increasing G_m^o. Thus, addition of DTT to sanguinarine-containing media (pH=7.4) produces the uncharged, reduced, relatively hydrophobic form that partitions into the membrane in quantities large enough to cause disruption of both lipid bilayer and red blood cell membranes. We tested this hypothesis that the uncharged forms of sanguinarine are responsible for the increase in G_m^o by making use of the acid-base properties of sanguinarine. Using a pK value of 8.5 (*see* Fig. 3) and the Henderson-Hasselbach equation, we calculated the concentration of the uncharged form of sanguinarine at the various experimental media sanguinarine concentrations and pH. Figure 9 is a log-log plot of G_m^o *vs.* total sanguinarine concentration at pH 7.4, having a slope of ≈4 and an r value >0.99. Inclusion of data for 5×10^{-5} M total sanguinarine at pH 8 and 1×10^{-5} M total sanguinarine at pH 10 is shown in Fig. 11. The correlation between G_m^o and total sanguinarine concentration is poor under these conditions. If, however, we plot the log of G_m^o *vs.* the log of the concentration of the uncharged form of sanguinarine at pH 7.4; 8 and 10 we find that the slope is ≈4 while $r>0.99$ (Fig. 12). Thus, using the concentration of the uncharged form of sanguinarine, all values for G_m^o, regardless of the pH at which measurements were made, fall on the same line. These results thus support

the hypothesis that it is the uncharged (relatively hydrophic) form of sanguinarine which is responsible for the increased ionic permeability of bilayers.

Discussion

The data described in this paper shows two effects of sanguinarine on the transport of Na and K across human red cell membranes. Upon exposure of red blood cells to sanguinarine, there is an immediate inhibition of the Na−K pump (Figs. 1 and 5). Some 30 to 60 min post-exposure, the red cells also exhibit large increases in the dissipative transport of Na and K (Figs. 1 and 5). Our results suggest that these two effects on red cell cation transport are caused by two different chemical forms of sanguinarine. The data summarized in Fig. 4 indicate that the oxidized, positively-charged form of sanguinarine inhibits the K−Na pump but does not affect the oubain-insensitive movements of K and Na. On the other hand, the fact that the reduced, uncharged from of sanguinarine produced rapid lysis of red cells suggested that these forms of the compound are responsible for increased downhill movements of K and Na induced by sanguinarine.

This interpretation of the results of the experiments on red cells is confirmed by observations of the increase in the electrical conductance of lipid bilayers produced by sanguinarine. The similarity in the effects of sanguinarine on both systems is illustrated by the similarity in time course, by the lack of an effect of oxidized (charged) form of sanguinarine on bilayer conductance, and by the observation that the reduced (uncharged) form of sanguinarine caused disruption of bilayers. Finally, experiments performed exposing bilayers to sanguinarine in media of varied pH show that it is the uncharged forms of the compound that are responsible for increasing bilayer conductance (Figs. 11 and 12). On the basis of this evidence, we conclude that the effects of sanguinarine upon conservative and dissipative fluxes of Na and K represent separate and distinct actions of the oxidized and reduced forms of the sanguinarine molecule, respectively.

With regard to the nature of the sanguinarine-induced conductance in bilayers, comparisons are instructive. In the presence of sanguinarine and filipin (Andreoli & Monahan, 1968; Cass, Finkelstein & Krespi, 1970) bilayers are unstable at relatively high resistance. Filipin-treated bilayers break at resistances below $10^6\ \Omega\,cm^2$ (Cass et al., 1970), while the sanguinarine-treated films are unstable at $Rm < 10^5\ \Omega\,cm^2$. In contrast, amphotericin B and nystatin-treated films are stable at Rm as low as $10^2\ \Omega\,cm^2$ (Andreoli & Monahan 1968). The instability of the filipin-treated films, based upon observations of poor ion selectivity, is attributed to the formation of large pores. Consistent with the above reasoning, sanguinarine-treated bilayers are more stable and exhibit only moderate cation selectivity ($t_{Na}/t_{Cl}=3$–4) while the nystatin and amphotericin B-treated membranes are very stable and anion selective ($t_{Cl}/t_{Na}=19$ (Andreoli & Watkins, 1973)). In contrast to nystatin and amphotericin B but similar to filipin, the effect of sanguinarine on bilayer conductane cannot be reversed by removing the compound from the membrane bathing solutions. These differences between uncharged sanguinarine and filipin, on the one hand, and amphotercin B and nystatin, on the other, may be due to the greater lipid solubility of the former compounds.

The above comparisons of sanguinarine with the polyene antibiotics illustrate a number of similarities between the compounds. While the nature of the sanguinarine-induced conductance pathway is unknown, it is well established that the polyene antibiotics are pore formers (Andreoli & Monahan, 1968; Holtz & Finkelstein, 1970; Cass et al., 1970; Andreoli & Watkins 1973; Andreoli, 1974; Holtz, 1974). This raises the possibility that sanguinarine also induces channels in bilayers.

The fact that bilayer conductance depends upon the 4th power of the sanguinarine concentration when the compound is symmetrically applied and the 2nd power upon asymmetric exposure is consistent with the idea that sanguinarine forms channels. Behavior of this type is similar to that observed with nystatin and amphotericin B (Finkelstein & Cass, 1968; Andreoli & Monahan 1968; Cass et al., 1970). Such high-power dependence of bilayer conductance on modifier concentration has also been reported for the voltage-dependent alamethecin (Eisenberg, Hall & Mead, 1973). In contrast, such results were not obtained in studies of carriers, e.g., valinomycin (Andreoli, Tieffenberg & Tosteson, 1967*b*; Cass et al., 1970) or peptide PV (Ting-Beall, Tosteson, Gisin & Tosteson, 1974).

Finally the steepness with which current rises as a function of applied voltage is consistent with the interpretation that sanguinarine is a voltage-dependent pore former. Sanguinarine-treated membranes exhibit an *e*-fold change in current for a 34 mV change in potential. Such a rapid rise in current, given that G_m^o depends upon the first power of the salt concentration and the uncharged form of the sanguinarine molecule, is highly suggestive that sanguinarine is a voltage-dependent pore former. There is no known carrier which produces an *e*-fold increase in membrane current for an increase in membrane potential difference of less than 50 mV. In contrast, membranes

treated with pore formers such as EIM (Ehrenstein, Lecar & Nossal, 1970), alamethicin (Eisenberg et al., 1973) and voltage-dependent anion-selective channels from paramecium mitochondria (Schein, Colombini & Finkelstein, 1976) all show *e*-fold ΔI for $\Delta V < 50$ mV. While the above do not constitute proof that sanguinarine is a voltage-dependent pore former, taken together the data favor such an interpretation.

The authors wish to thank Dr. James E. Hall for helpful discussions and Ramon Latorre for his comments on the manuscript.

This work was supported by NIH Grant HL-12157 to D.C. Tosteson and also NIH Postdoctoral Fellowship HL-01672 and NIH Grant HL-21179 to P.M. Cala. J.G. Nørby was the recipient of a travel grant from Fonden til Laegevidenskabens Fremme, Denmark.

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Received 2 February 1981; revised 16 June 1981