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SURAMIN: A POTENT ATPase INHIBITOR WHICH ACTS ON THE INSIDE SURFACE OF THE SODIUM PUMP

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SUMMARY

(1) The impermeant polyanion suramin inhibits the (Na^+–K^+)-activated ATPase in broken membrane preparations. Suramin does not affect Na^+ or K^+ transport in intact red cells or resealed red cell ghosts when present in the incubation medium, but appears to inhibit the Na^+ pump in ghosts resealed with suramin inside. Other ATP-requiring enzymes are also inhibited by suramin with similar potency (50% inhibition at 10^{-5}–10^{-4} M) suggesting an interaction with the substrate site of the enzyme.

(2) K^+-activated *p*-nitrophenylphosphatase activity and [^{3}H]ouabain binding are also inhibited by suramin.

(3) The inhibition by suramin is reversed by washing the membranes.

(4) It is concluded that suramin inhibits the Na^+–K^+ transport system through interactions with the intracellular part of the pump.

(5) The potential usefulness of suramin as a pump inhibitor and the possibility of modifying its structure to yield fluorescent derivatives are discussed.

INTRODUCTION

The trypanocidal drug suramin has been shown to be a potent inhibitor of some hydrolytic and oxidative enzymes (*e.g.* see Wills and Wormall[1]), but does not cross yeast[2] or red cell membranes[3]. Since suramin was reported to inhibit Na^+-stimulated ATPase from guinea pig brain[4] and it resembles certain fluorescent probes in its constituent naphthalene trisulphonate groups (Fig. 1) we investigated

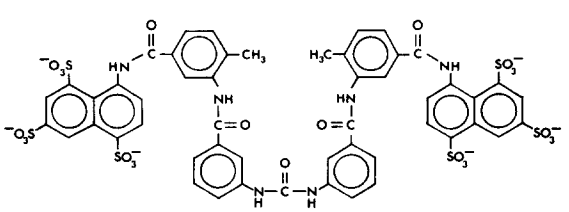

Fig. 1. Suramin. *sym*-bis(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamino-4,6,8-trisulphonate) carbamide, used as the hexasodium salt.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

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its effect on (Na^+-K^+)-activated ATPase, in view of the potential usefulness of site-specific, spectroscopically active inhibitors of the Na^+-K^+ pump.

In the present work we describe the action of suramin on (Na^+-K^+)-activated ATPase, *p*-nitrophenylphosphatase and ouabain binding in red cell ghosts. Since suramin inhibits all these parameters associated with the Na^+ pump in broken membrane preparations, its action on cation fluxes in intact red cells and resealed ghosts was also studied. Suramin has no effect on Na^+ and K^+ fluxes when present in the incubation medium. It is concluded that suramin is a potent inhibitor of the Na^+ pump and that its site of action is located at the intracellular membrane surface.

MATERIALS AND METHODS

Preparation of red cell membranes

Blood drawn into acid citrate–dextrose medium was obtained from the Blood Bank. The red cells were washed three times by centrifugation in isotonic NaCl medium buffered with 20 mM Tris (pH 7.5 at 20 °C) and lysed in 14 vol. of 1 mM EDTA, 10 mM Tris, pH 7.5 at 4 °C. The membranes were washed twice by centrifugation ($25000 \times g$, 10 min) and a further two times in 10 mM Tris (pH 7.5 at 4 °C) before storage at −20 °C. Before use, the thawed ghosts were washed once in 0.1 mM EDTA, 10 mM Tris, pH 7.5 at 4 °C, and resuspended in a volume of 10 mM Tris (pH 7.5 at 37 °C) equivalent to the original packed cells.

(Na^+-K^+)-activated ATPase determination

Red cell membranes (approx. 3 mg protein/ml) were incubated for 1 h in a medium containing 1.25 mM Na_3ATP, 140 mM NaCl, 1.5 mM $MgCl_2$, 20 mM Tris (pH 7.5 at 37 °C) either in the presence or absence of 10 mM KCl, or in the presence of 10 mM KCl with and without 0.1 mM ouabain. Assays were run in triplicate. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5%, and the precipitate removed using an Eppendorf 3200 microcentrifuge. Aliquots of supernatant were assayed for phosphate by a modification of the method of Weil-Malherbe and Green[5]. Protein was determined by a modification of the biuret method. (Na^+-K^+)-activated ATPase was calculated as either the ouabain-sensitive difference in ATP hydrolysis, or the increase in ATPase when 10 mM KCl was added to the medium.

p-Nitrophenylphosphatase determination

p-Nitrophenylphosphatase was measured in red cell ghosts (approx. 5 mg protein/ml) suspended in a medium containing 2.5 mM $MgCl_2$, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), 20 mM Tris (pH 7.5 at 37 °C) and 4 mM ditris *p*-nitrophenyl phosphate. Assays were run in triplicate, K^+ activation was measured by the presence or absence of 50 mM KCl. After a 1 h incubation the reaction was stopped with trichloroacetic acid as before, and after centrifugation a 0.5-ml aliquot was diluted with 3 ml of 0.2 M NaOH and absorbance read in the spectrophotometer at 410 nm.

Ouabain binding

[^{3}H]Ouabain (New England Nuclear NET-211) was evaporated to dryness in

an atmosphere of N_2 and redissolved with a 10-fold dilution by carrier ouabain. The incubation medium contained 8 nM [^{3}H]ouabain, and red cell ghosts (approx. 3 mg protein/ml) with either 1 mM Na_3ATP, 2 mM $MgCl_2$, 100 mM NaCl, 10 mM Tris (pH 7.5 at 37 °C) or 5 mM phosphate, 14 mM Tris (pH 7.5 at 37 °C), 3 mM $MgCl_2$. In order to estimate the amount of non-specific [^{3}H]ouabain bound some tubes were incubated with the addition of unlabelled ouabain to a final concentration of 0.1 mM. At zero time, and appropriate time intervals, 3-ml aliquots were taken and the membranes washed 3 times by centrifugation (25000 × g, 10 min) in 40 ml of a medium containing 50 mM NaCl, 0.01 mM ouabain, 10 mM Tris (pH 7.5 at 4 °C). The final pellet was resuspended with vigorous mixing in 1 ml of ethanol, 14 ml of Bray's solution added, and counted for ^{3}H with automatic external standardization for quench correction. Bound ouabain was calculated after correcting for binding in the presence of excess unlabelled ouabain, which normally represented about 8% of the total counts.

Preparation of hypotonically-resealed ghosts

Freshly-drawn red cells were washed 3 times as before, and finally packed at 25000×*g* for 10 min. The packed cells were lysed in 10–15 vol. of an ice-cold solution containing 4 mM $MgCl_2$, 3 mM Na_3ATP. After 5 min 150 mM NaCl (containing ^{24}NaCl) was added to a final total Na^+ concentration of 10 mM, and after 10 min 1 M KCl or choline chloride was added to a final concentration of 50 mM. The ghosts were then resealed by incubation at 37 °C for 40 min, and finally washed 6 times by centrifugation (15000×*g*, 5 min) in a medium containing NaCl. 75 mM, 15 mM Tris (pH 7.7 at 37 °C).

Measurement of K^+ and Na^+ fluxes

K^+ influx and Na^+ efflux were measured by methods similar to those of Glynn[6]

MATERIALS

Suramin ("antrypol") was a gift from ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire. ATP (trisodium salt), *p*-nitrophenyl phosphate ditris salt, and ouabain were obtained from Sigma Chemical Co., St. Louis, Mo. Choline chloride was recrystallised from hot ethanol.

RESULTS

Fig. 2 shows the effect of suramin on the (Na^+–K^+)-activated ATPase activity of human red cell ghosts. The concentration necessary for 50% inhibition is about 50 μM. In spite of the apparent high affinity, the inhibition is reversible since it disappears after washing the membranes that have been exposed to suramin concentrations up to 1 mM (Table I). The inhibitory action of suramin is observed in (Na^+–K^+)-ATPases prepared from different tissues (Table II) and appears to be related to the enzyme site that interacts with ATP, since ouabain-insensitive ATPase and other ATP-requiring enzymes are also inhibited by suramin with similar potency (Table II and Fig. 3). Experiments with suramin at varying ATP concentrations on the ouabain-sensitive and ouabain-insensitive components of the red cell ATPase give mixed-type inhibition, suggesting a complex interaction. Suramin does not inhibit the cholin-

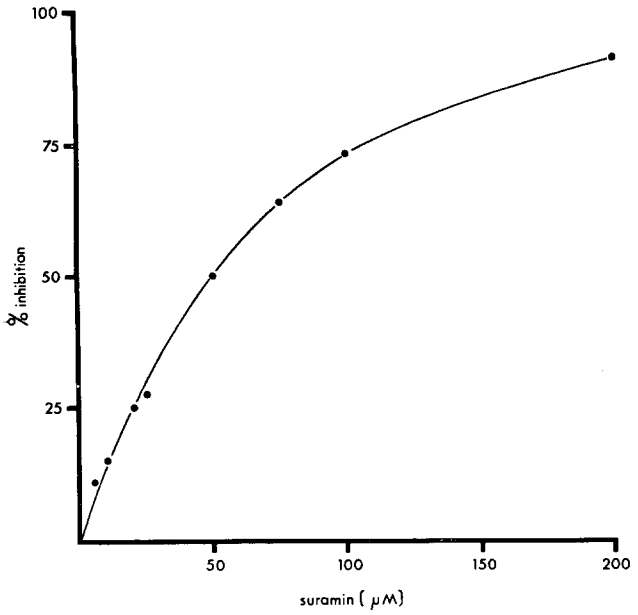

Fig. 2. The effect of suramin on the (Na^+–K^+)-activated ATPase activity in human red cel ghosts. Experimental conditions: Red cell ghosts (approx. 3 mg protein/ml) were incubated at 37 °C for 1 h in 1.4 ml of a medium containing 1.25 mM Na_3ATP, 140 mM NaCl, 1.5 mM $MgCl_2$, 20 mM Tris (pH 7.5 at 37 °C) ± 10 mM KCl. Acitivity of the uninhibited preparation was 284 nmoles P_i/mg protein per h measured as the 10 mM KCl-dependent stimulation. Points represent the mean of triplicate determinations. Three separate experiments, and two using ouabain instead of removing 10 mM KCl gave similar results.

esterase activity assayed in the same preparation of eel electric organ that contained the inhibited (Na^+–K^+)-activated ATPase.

If the inhibition by suramin is not a consequence of a general deleterious effect of the large polyanion on the membrane or the ATPase, but is caused by an interaction at a specific site. In intact cells which are impermeable to suramin[3] inhibition may depend on which side of the membrane is exposed to the drug. If suramin does interact at the ATP site, which is intracellular, no inhibition should be observed when intact cells are exposed to suramin in the medium. These predictions were tested by assaying the effect of suramin on pump fluxes in intact and resealed human red cells.

When suramin is present in the medium, concentrations as high as 1 mM have no effect on the pump Na^+ and K^+ fluxes in either intact red cells or hypotonically resealed ghosts (Table III). This is in contrast with its effect in the broken membrane preparations where concentrations 100 times smaller cause significant inhibition of the (Na^+–K^+)-activated ATPase (Fig. 2).

This suggests that the site of action of suramin is indeed intracellular. We attempted to incorporate suramin inside the cells by resealing ghosts lysed in the presence of suramin. This poses several technical problems since suramin is known to bind strongly to proteins, including red cell haemolysates[2,3]. Our own experiments have shown that haemoglobin protects against inhibition of the red (Na^+–K^+)-

TABLE I

REVERSIBILITY OF SURAMIN INHIBITION OF RED CELL MEMBRANE ATPase AND NITROPHENYLPHOSPHATASE ACTIVITY

Red cell ghosts were incubated at 2 mg protein/ml for 15 min, in 10 mM Tris (pH 7.5 at 37 °C) with and without suramin. Samples were washed 3 times by centrifugation ($25000 \times g$; 10 min) in 40 vol. Tris medium, and assayed for ATPase or p-nitrophenylphosphatase.

	ATPase activity (nmoles P_i/mg protein per h)*				*p-Nitrophenylphosphatase activity** (nmoles p-nitrophenyl phosphate/mg protein per h)*			
	Initial activity		*Activity after 3 washes*		*Initial activity*		*Activity after 3 washes*	
	Ouabain-sensitive	*Ouabain-insensitive*	*Ouabain-sensitive*	*Ouabain-insensitive*	*K^+-dependent*	*K^+-independent*	*K^+-dependent*	*K^+-independent*
Control	278	296	237	220	18.1	45.0	16.3	41.2
0.1 mM suramin	47	71	234	201	—	—	—	—
1.0 mM suramin	8	17	208	206	2.7	8.7	12.6	39.3

* Conditions as in Fig. 2.
** Conditions as in Fig. 6.

TABLE II

INHIBITION BY SURAMIN OF ATP-REQUIRING ENZYMES

Enzyme	*Concentration of suramin for 50% inhibition* (μM)*
(Na^+–K^+)-activated ATPase from:	
human red cell ghosts	50
llama red cell ghosts	35
electric-eel microsomes**	80
Ouabain-insensitive red cell ATPase	15
Luciferase (firefly)***	6
Hexokinase (data from Wills and Wormall[1])	33 (for 75% inhibition)

* These are approximate values since the degree of inhibition at a certain concentration of suramin depends on the amount of protein or membranes present.
** Electric eel microsomes were prepared by a modification of the method of Glynn[17].
*** Luciferase was assayed by the method of Slayman (personal communication).

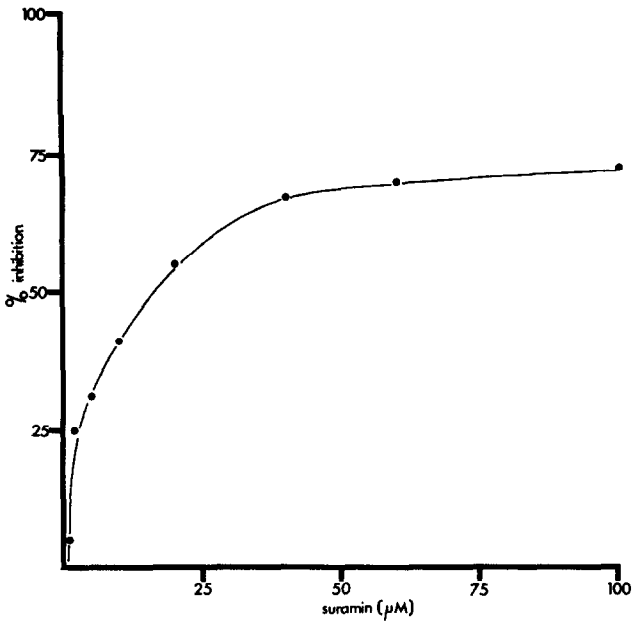

Fig. 3. The effect of suramin on the ouabain-insensitive ATPase activity in human red cell ghosts. Red cell ghosts (approx. 3 mg protein/ml) were incubated at 37 °C for 1 h in 1.4 ml of a medium containing 1.5 mM $MgCl_2$, 140 mM NaCl, 125 mM Na_3ATP, 20 mM Tris (pH 7.5 at 37 °C) Activity of the uninhibited preparation was 391 nmoles P_i/mg protein per h. Points represent the mean of triplicate determinations.

activated ATPase by 200 μM suramin probably by decreasing the concentration of free inhibitor, which is adsorbed to the protein. Thus, high concentrations of suramin must be included in the haemolysing medium to achieve sufficient intracellular concentrations of free inhibitor to observe an effect. However, high suramin concentrations (1 mM) prevent resealing of the membrane. Even at 50 μM suramin the fraction of resealed ghosts decreases about 50% with respect to the controls.

Nevertheless, suramin appears to inhibit ouabain-sensitive Na^+ efflux into Na^+ media containing either 0 or 10 mM K^+ in ghosts haemolysed and resealed in the presence of 50 μM suramin nominal concentration. The magnitude of the effect is variable; the maximum inhibition observed under these conditions was about 40%, but no inhibition was observed in one experiment.

It therefore appears that suramin interacts with the part of the sodium pump that faces the inside of the cell, *i.e.* the ATP and Na^+-binding sites. Attempting to investigate the mechanism of inhibition by suramin we studied its effect on ouabain binding and *p*-nitrophenylphosphatase.

Although cardiac glycosides bind to the external part of the pump[7,8] the rate and extent of binding depend on the presence of effectors that interact with the internal part of the pump, *e.g.* Mg^{2+}, P_i, ATP, Na^+ (refs 9–14).

Suramin, acting on the inside, could alter the binding of effectors and/or their reactions with the pump and this should be reflected by alterations of ouabain binding on the outside. Fig. 4 shows the time course of ATP or P_i-promoted ouabain

TABLE III

THE EFFECT OF EXTRACELLULAR SURAMIN ON K^+ INFLUX IN INTACT HUMAN RED CELLS AND Na^+ EFFLUX FROM RESEALED HUMAN RED CELL GHOSTS

K^+ influx into intact cells was measured in a medium containing 140 mM NaCl, 8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM Tris (pH 7.5 at 37 °C) in the presence and absence of $5 \cdot 10^{-5}$ M ouabain, and ±1 mM suramin. Na^+ efflux from hypotonically-resealed ghosts was measured into either 75 mM NaCl, 5 mM Tris (pH 7.5 at 37 °C) or 65 mM NaCl, 10 mM KCl, 5 mM Tris (pH 7.7 at 37 °C) in the presence and absence of $5 \cdot 10^{-5}$ M ouabain and 1 mM suramin. 1 h incubation at 37 °C.

	K^+ influx in intact red cells (mmoles/l cells per h)			*Na^+ efflux from hypotonically-resealed red cell ghosts (% isotope lost/h)*					
				75 mM NaCl medium (Na^+–Na^+ exchange)			*65 mM NaCl, 10 mM KCl in medium (Na^+–K^+ exchange)*		
	Total	*+ Ouabain*	*Ouabain-sensitive difference*	*Total*	*+ Ouabain*	*Difference*	*Total*	*+ Ouabain*	*Difference*
Control	2.46	0.61	1.75	29.1	10.3	18.8	38.6	10.5	28.1
1.0 mM suramin	2.49	0.63	1.76	31.4	12.3	19.1	39.9	12.6	27.2

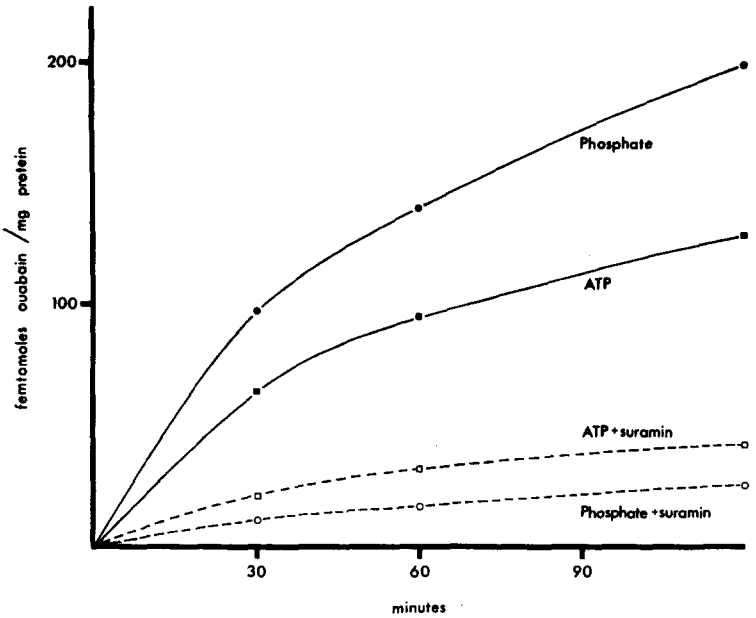

Fig. 4. The effect of suramin on the rate of ATP and P_i-dependent ouabain binding to human red cell ghosts. Experimental conditions: 8 nM [^{3}H]ouabain with 1 mM Na_3ATP, 2 mM $MgCl_2$, 100 mM NaCl, 10 mM Tris (pH 7.5 at 37 °C) or 5 mM phosphate, 14 mM Tris (pH 7.5 at 37 °C) 3 mM $MgCl_2$. Suramin concentration 0.5 mM. ■—■, ATP; □—□, ATP+suramin; ●—●, P_i; ○—○, P_i+suramin. Ouabain concentration 8 nM.

binding to red cell membranes in the presence and absence of suramin. There is a large inhibition of both ATP and P_i-promoted ouabain binding. The inhibition is in the amount of ouabain bound, the proportional rate of binding is unaffected by suramin. This is further demonstrated in Fig. 5 where a constant degree of inhibition is still observed after 4.5 and 6 h of incubation in the presence of P_i at any suramin concentration. It may be interesting to note that the amount of ouabain bound in the presence of P_i, which is larger than that bound with ATP[11,12], is inhibited by suramin to a greater extent than ATP-promoted ouabain binding (Fig. 4).

Another interesting feature of the inhibition of ouabain binding by suramin is an apparent maximal effect that is less than 100% (Fig. 5).

The similarity between the dose-response curves for suramin inhibition of ouabain binding (Fig. 5) and (Na^+–K^+)-activated ATPase (Fig. 2) lends further support to the idea that inhibition of ouabain binding by suramin results from the interaction of the latter with the inside part of the pump. Also, a direct interaction of suramin with ouabain binding sites on the outer surface is inconsistent with the absence of suramin effects on Na^+ and K^+ fluxes in intact or reconstituted red cell membranes, which show normal ouabain sensitivity in the presence of 1 mM suramin (Table III). Since ouabain binding is decreased by suramin, and ouabain sensitivity is used in some experiments as a criterion to define functions related to the sodium pump, experiments on the ATPase and *p*-nitrophenylphosphatase were carried out using K^+ activation as well as ouabain inhibition as criteria for assessing suramin inhibition of Na^+ pump functions, both methods yielding identical results.

Further evidence of suramin acting on the intracellular part of the pump arises from its effect on K^+-activated *p*-nitrophenylphosphatase activity. *p*-Nitrophenylphosphatase activity has been associated with the (Na^+–K^+)-activated ATPase owing to its ouabain sensitivity. It has been suggested that the substrate site is intracellular, whereas the K^+ activation occurs at the outer surface[15], although this is still controversial[16]. Fig. 6 shows that suramin also inhibits the K^+-activated *p*-nitrophenylphosphatase, with a similar concentration dependence to its effect on ATPase and ouabain binding. The inhibition is at least partly reversible (Table I) as in the case of (Na^+–K^+)-ATPase. Since external suramin has no effect on the K^+ stimulation of the pump (Table III), it is unlikely that suramin interacts with the K^+ site of *p*-nitrophenylphosphatase, located on the outside.

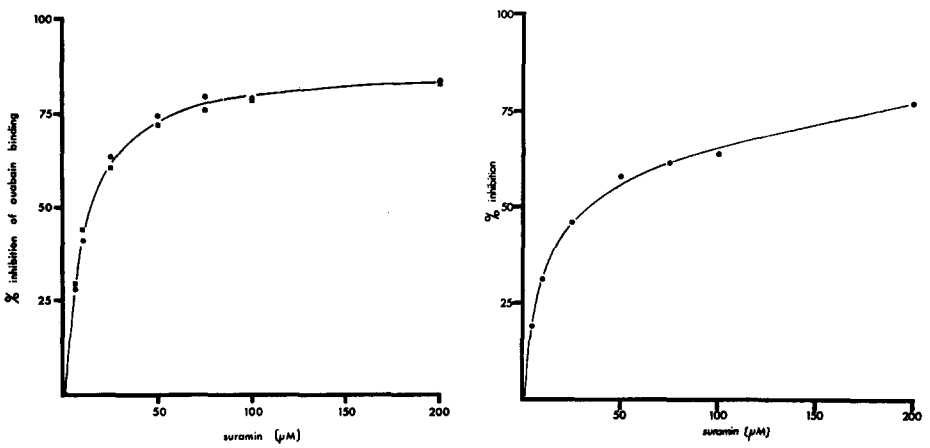

Fig. 5. The effect of varying concentrations of suramin on P_i-dependent ouabain binding to human red cell ghosts. Experimental conditions as in Fig. 4. Bound ouabain in the absence of suramin was 550 femtomoles/mg protein. ●—●, 4.5 h incubation; ■—■, 6 h incubation. Ouabain concentration 8 nM.

Fig. 6. The effect of suramin on the K^+-dependent, *p*-nitrophenylphosphatase activity in human red cell ghosts. Experimental conditions: 2.5 mM $MgCl_2$, 1 mM EGTA, 20 mM Tris (pH 7.5 at 37 °C), 4 mM ditris *p*-nitrophenyl phosphate, with and without 50 mM KCl. Activity of the uninhibited preparation was 21 nmoles *p*-nitrophenol/mg protein per h.

DISCUSSION

The main finding in this paper is that suramin inhibits the sodium pump through an interaction of the drug with a site or sites located at the inside membrane surface.

The inhibition of the (Na^+–K^+)-activated ATPase, and the failure of high concentrations of suramin to inhibit transport in intact cells when applied externally provide strong evidence in support of this hypothesis, although the inhibition of Na^+ pumping by suramin incorporated inside resealed ghosts is difficult to observe and reproduce because of technical problems. The observation that other ATP-requiring enzymes are also inhibited by suramin suggests that the ATP-binding site may be involved in the mechanism of inhibition. However, the ouabain binding and

p-nitrophenylphosphatase experiments indicate that the inhibitor also affects, directly or indirectly, other functions of the ATPase. It has been shown that glycoside binding does not necessarily involve phosphorylation of the enzyme[13], nor is it specific for ATP since other nucleotides are equally effective[9,10,14]. Therefore the decreased ouabain binding in the presence of suramin reflects combination of the inhibitor with the enzyme on the inside of the cell that need not be directly related to ATP binding or hydrolysis, but prevents ouabain binding at the outer surface.

The present results suggest that suramin may be a useful tool in the investigation of the mechanisms involved in cation translocation and ATP hydrolysis. Its high affinity for ATPases and the sidedness of its inhibitory effects offer possibilities for studies of partial reactions in ATP hydrolysis and cation pumping.

Another potential use for suramin is in the design of spectroscopically active inhibitors of the inward-facing part of the Na^+ pump. Some other compounds which show interesting spectral properties and may act at the inside site as inhibitors include a sulfhydryl analog of ATP[18], a fluorescent analog of adenine nucleotides[19], and the hallucinogen harmalin[20], which is fluorescent. Suramin absorbs strongly in the ultraviolet region; its extinction coefficient is about $2.8 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 313 nm, and it shows fluorescence with a maximum around 400 nm, although the quantum yield appears to be very low, and the possibility of the fluorescence arising from traces of a contaminant has not yet been completely ruled out. Nevertheless, the similarity between the structure of the naphthalene sulfonate fluorescent probes[21,22] and suramin (Fig. 1) suggests the possibility of modifying suramin to obtain a more spectroscopically useful molecule.

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