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ACCUMULATION OF VANADIUM BY TUNICATE BLOOD CELLS OCCURS VIA A SPECIFIC ANION TRANSPORT SYSTEM

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Tunicates, or sea squirts, are known to sequester vanadium to very high concentrations within specialized blood cells. They selectively accumulate the element from seawater against a 10^6- to 10^7-fold concentration gradient, and store it mainly as V(III). The mechanism for this selective accumulation involves the facilitated diffusion of vanadate across the blood cell plasma membrane followed by intracellular reduction to a non-transportable cation. Evidence for this mechanism was obtained by studying vanadate and [^{48}V]vanadate influx into living blood cells (vanadocytes). Influx of [^{48}V]vanadate into the cells is a rapid ($t_{1/2}$ = 57 s at 0°C) process which can be saturated (K_m = 1.4 (± 2%) mM). Net vanadate accumulation is equal to isotopic influx, and accumulated vanadate is not released by washing cells with EDTA. Uncouplers of oxidative phosphorylation and glycolytic inhibitors have no effect on the rate of influx. Phosphate competes with vanadate for transport, and is itself taken up by the cell. The similar anions, sulfate and chromate, neither inhibit transport, nor are they taken up by the vanadocyte. Influx is inhibited by those stilbene disulfonate derivatives known to bind specifically to the external transport site of the anion exchange protein in the human erythrocyte membrane. During the influx of vanadate, the electron paramagnetic resonance (EPR) signal of intracellular vanadyl increases, indicating that transported V(V) is reduced upon entering the cell. The EPR signal of the blood cells at room temperature is characteristic of unbound V(IV), in agreement with reports that reduced vanadate is not bound to a protein or other macromolecule in these cells.

Introduction

It has been known for some time that the tunicate, a common marine protochordate, concentrates vanadium within specialized blood cells [1,2], but neither the mechanism of accumulation nor its purpose is yet understood. In this paper we present evidence that the selective accumulation of vanadium by the tunicate, *Ascidia nigra,* is mediated by a specific anion transport system, with subsequent reduction of intracellular vanadate to V(IV). In several respects, this process resembles that previously observed for vanadium uptake by human erythrocytes [3–6].

In seawater, vanadium exists as the V(V) vanadate anion, $H_2VO_4^-$ [7], at a concentration of approximately $5 \cdot 10^{-8}$ M [8]. In the vanadium-containing blood cell, the vanadocyte, the concentration of vanadium can be as high as 0.15 M [9]. In many species, including *A. nigra,* the intracellular vanadium is mainly in the V(III) state, with up to 10% in the V(IV) state [10,11]. Therefore, the cell accumulates vanadium against at least a six orders of magnitude concentration ratio. The storage of the metal in the

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; CCMP, carbonylcyanide *p*-trifluoromethylphenylhydrazone; Hepes; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

unstable +3 valence state indicates the presence of an acidic, reducing environment within the vanadocyte.

A mechanism linking transport and reduction of vanadate has been proposed [12]. The mechanism requires a transport site that is specific for metal-containing oxyanions; only vanadate and chromate exist as such in seawater. To achieve the high concentration ratio, it has been proposed that the anionic vanadate is reduced upon entering the cell. This step produces a cation to which the cell membrane is assumed to be impermeable, and lowers the intracellular pH. It was suggested that the production and maintenance of the extreme concentration gradient across the plasma membrane does not require direct coupling to catabolism, but rather depends only on the concentration of the intracellular reducing agent.

We now report evidence in support of this hypothesis. We have investigated the influx of vanadate into the blood cells of *A. nigra.* Phosphate and sulfate influx were studied to determine the specificity of transport, and to determine the inhibitory effects of these oxyanions and chromate upon vanadate influx. The rate of influx of vanadate was measured in the presence of metabolic inhibitors and inhibitors of anion transport. Electron paramagnetic resonance (EPR) spectroscopy was used to follow changes in the concentration of reduced vanadium within tunicate blood cells exposed to vanadate. This report will show that accumulation of vanadium by tunicate blood cells is similar to that in erythrocytes, in that transport occurs through an anion channel and is followed by a non-enzymatic reduction. This mechanism accounts for sequestration of vanadium in the tunicate *A. nigra.*

Experimental Procedures

Materials

Radioisotopic vanadate in the form of [^{48}V]-vanadyl chloride and Aquasol Scintillation fluid were purchased from Amersham, Chicago, IL. [^{32}P]-Phosphate and [^{35}S]sulfate were obtained from New England Nuclear Corp., Boston, MA. DIDS was obtained from Pierce Chemical Co., Rockford, IL, dissolved in deoxygenated buffer and frozen in small aliquots until used. DNDS was purchased from Aldrich Chemical Co., Milwaukee, WI, and recrystallized twice from doubly distilled water. Sodium phosphates, sodium sulfate, sodium fluoride and sodium vanadate were purchased from Fisher Scientific Co., Pittsburgh, PA. DL-Dithiothreitol, 2,4-dinitrophenol, Hepes and CCMP were purchased from the Sigma Chemical Co., St. Louis, MO. Eosin-5-maleimide was purchased from Molecular Probes, Inc. All other chemicals were obtained from Baker Chemical Co., Phillipsburg, NJ.

Methods

Atomic emissions measurements were performed on a Spectroscan III échelle d.c. argon plasma emission spectrometer. Electron paramagnetic resonance spectra were recorded on a custom-built spectrometer based upon a Varian V-4500 system, utilizing a Varian V4012A-HR 12-inch magnet, microwave circulator, Schottky diode detector (Micro-Now, Ltd.), a 100 kHz modulation unit and preamplifier and a Varian E23-1 cavity. A Varian flat quartz cell was used for room-temperature measurements. Low-temperature measurements were carried out in liquid nitrogen using a quartz dewar and 3-mm quartz tubes. [^{32}P]Phosphorus and [^{35}S]sulfur activities were measured to ±1% precision with a Beckman LS-100c liquid scintillation system. Samples containing [^{48}V]-vanadium were dried on planchettes and activity determined with a Baird Atomic Model 530 Spectrometer and gas-flow counter. Cell counts were made using a Levy-Hausser hemocytometer.

Preparation of cell suspension.

Tunicates were obtained from Key Biscayne, FL, and maintained in salt-water aquaria at 23°C as described previously [13]. Blood was obtained via heart puncture. After centrifugation at 2500 rev./min for 3 min, the plasma was discarded. The cells were washed twice with ice-cold, isotonic, iso-osmotic Hepes/NaCl buffer (50 mM Hepes/0.50 M NaCl, pH 8.0), then resuspended in the buffer, stored at less than 1°C and used within 1 h. Aliquots were diluted 100 to 200-fold with cold 5 mM dithiothreitol in Hepes/NaCl for determining the cell count.

Influx experiments

A stock solution of 5 mM sodium vanadate in Hepes/NaCl buffer was briefly heated in a boiling water bath to degrade polymers [14–17]. Radioisotopic solutions were prepared by adding equal

volumes of [^{48}V]vanadyl chloride in 1 M HCl and 1 M NaOH to the stock solution of vanadate, again heating to remove polymers (air oxidation of V(IV) to V(V) takes place over several minutes [18]).

All influx experiments were initiated by addition of the [^{48}V]vanadate, [^{32}P]phosphate or [^{35}S]-sulfate solution to a cell suspension to give a final concentration of $1 \cdot 10^8$ to $3 \cdot 10^8$ cells per ml, followed by mixing. The cell suspension was kept in a thermostatically controlled shaking water bath during the reaction. At timed intervals, 0.10–0.20 ml samples of the cell suspension were withdrawn and influx stopped by centrifugation in a Beckman microfuge for 20 s. The concentration of the extracellular radioisotope was determined by counting 50–100 µl of the supernatant. The total concentration of [^{48}V]-vanadate in the reaction mixture was determined by counting an uncentrifuged aliquot. When the effect of an anion transport inhibitor, metabolic inhibitor or another oxyanion upon influx was being investigated, the reagent was added to the cell suspension (20°C) 20 min before addition of the radioisotopic solution. All experiments were performed twice, and the results averaged.

To determine the net uptake of vanadate, cells were incubated with vanadate for 15 min at room temperature prior to centrifugation. This period had been determined to be sufficient to allow equilibration of [^{48}V]vanadate into the cells under similar conditions. In one experiment, EDTA was added to the cell suspension prior to centrifugation, to release any vanadate bound to the cell surface or to the interior of the tube. The concentration of vanadium in the supernatant was found by comparing the intensity of vanadium atomic emission line at 4379 Å to that of known concentrations of vanadate in the same matrix. The off-line intensity was also measured to correct for signal enhancement due to the salt content of the buffer.

EPR measurements

A calibration curve relating the intensity of the +3/2 line of the room temperature V(IV)aq. spectrum to the concentration of vanadyl sulfate in acidic solution was prepared [19]. Spectra were recorded at 9.55 GHz and 9 MW power. Before each experiment, a room-temperature spectrum of the cell suspension was recorded at the same frequency and power settings, to provide a measure of the V(IV) present in the cells. To prepare a time course of the reduction of V(V) to V(IV), the remainder of the cell suspension was divided into aliquots, and a known concentration of vanadate or buffer added. The reaction mixture was transferred to a quartz EPR tube and flushed with nitrogen. At timed intervals, the reaction was quenched by freezing the tube in liquid nitrogen. The relative increase in V(IV) concentration in the cells was calculated by comparing the peak-to-peak height of the 77 K spectra to that of cells with no added vanadate.

Results

Uptake of [^{48}V]vanadate by tunicate blood cells

The influx of anionic vanadate was determined by monitoring the concentration of [^{48}V]vanadate in the external solution of a 10% cell suspension ($1 \cdot 10^8$ cells/ml). A typical time course for vanadate influx, using unfractionated blood cells in Hepes/NaCl buffer, is shown in Fig. 1a. Accumulation of vanadate is monophasic to within 85% of attainment of a steady state of extracellular [^{48}V]vanadate concentrations. At 0°C, the half-time for influx is 57 s. From the temperature dependence of influx, displayed in an Arrhenius plot (Fig. 1b), the energy of activation of this transport process is approx. 8.5 kcal/mol.

If influx of vanadate is mediated by a limited number of transport sites, the rate of influx will approach a limit as those sites become saturated at high concentrations of vanadate. An approach to saturation is detected at initial concentrations of [^{48}V]vanadate greater than 0.40 mM for a $1 \cdot 10^8$ cells/ml suspension at 0°C (Fig. 2a). A Lineweaver-Burke plot of these data gives the Michaelis-Menten constants for the transport process; $K_m = 1.4$ mM, $V = 0.57$ mM $\cdot$ min^{-1} (rel. error = ±2%) (Fig. 2b).

The accuracy of these values could in principle be improved by measuring initial rates at higher concentrations of vanadate. However, at vanadate concentrations greater than 1 mM, increasing polymerization to the trimer, $V_3O_9^{3-}$, introduces significant error into the data, making this region inaccessible [14–17]

In the absence of net accumulation of [^{48}V]vanadate by tunicate blood cells, the maximum decrease in extracellular vanadate for an experiment such as

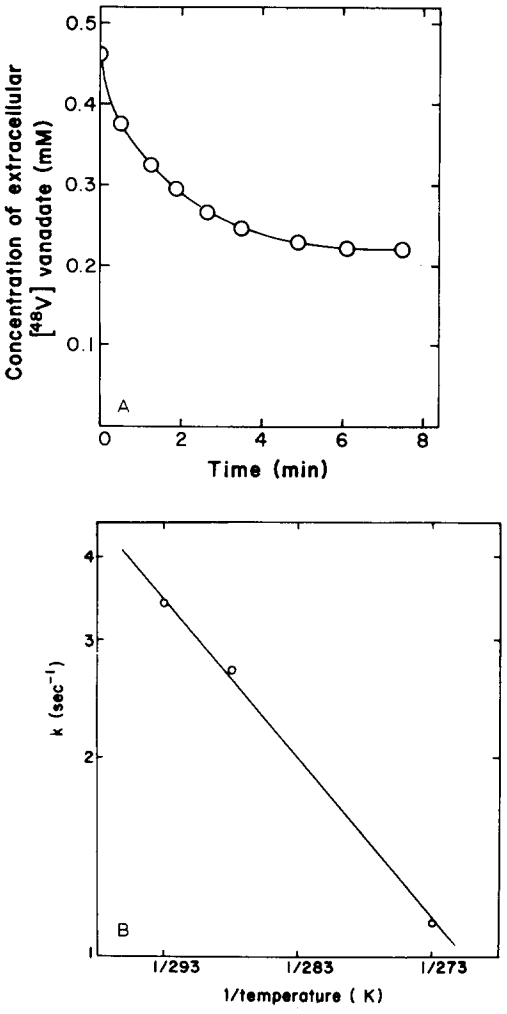


Fig. 1. (a) Uptake of [^{48}V]vanadate by unfractionated tunicate blood cells. 0.46 mM [^{48}V]vanadate was added to a 10% suspension of washed cells in 50 mM Hepes/0.50 M NaCl at pH 8.0 and incubated with shaking in an ice bath. The final concentration of [^{48}V]vanadate in the supernatant was 0.22 mM. A computer fit of the data gives $t_{1/2}$ = 57 s. (b) Arrhenius plot of [^{48}V]vanadate influx data. Separately prepared 10% suspensions of washed cells maintained in a thermostated shaking water bath were made 0.50 mM in [^{48}V]vanadate. The rate of influx of vanadate was measured as described for three suspensions at each temperature and averaged results are given. E_A = 8.48 kcal/mol.

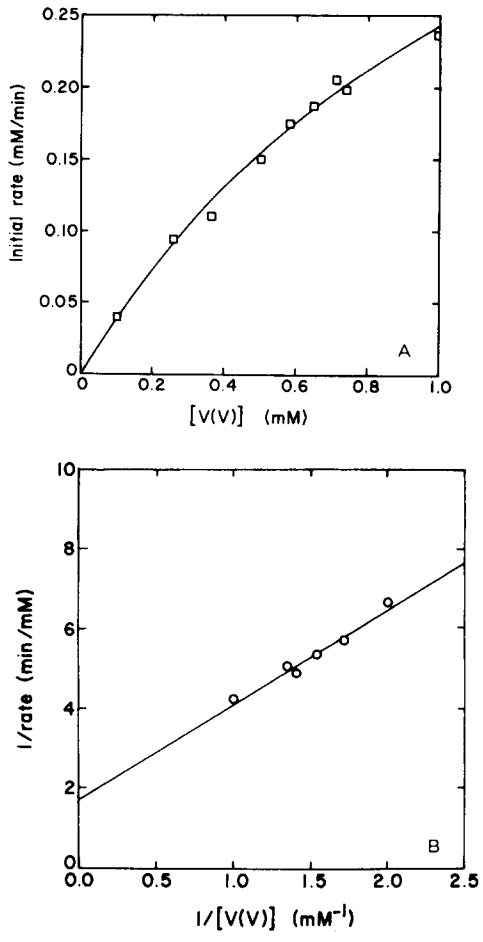


Fig. 2. (a) The rate of influx of [^{48}V]vanadate at 0°C vs. the experimentally determined concentration of [^{48}V]vanadate in 1 · 10^8 cells/ml suspensions at zero time. Corrections were made for variation in the concentration of cells in separate preparations and experiments were done in duplicate. (b) Lineweaver-Burk plot of data from (a). A least-squares fit of the data gives K_m = 1.4 mM and V = 0.57 mM · min^{-1}; rel. error = ±2%.

that shown in Fig. 1a, would be about 10% at equilibrium. From Fig. 1a, it can be seen that 47% of the exogenous vanadate is accumulated by the cells. Either the cells have accumulated vanadate from the external medium against a concentration gradient, or there is an exchange of external [^{48}V]vanadate with the highly concentrated vanadium already present in the blood cells. A determination of the extracellular vanadium concentration after the attainment of the steady state allows us to discriminate between these two possibilities.

In three separate experiments it was determined by atomic emission measurements that there was a net decrease in the external vanadium concentration equal to the observed decrease in external [^{48}V]-vanadate (Table I). When a 5-fold excess of EDTA

was added to the cell suspension after incubation with vanadate, the net decrease in extracellular vanadate persists. This result eliminates the possibility that vanadate is binding nonspecifically to the plasma membrane, or to the walls of the reaction vessel. If vanadate were so bound, it would be scavanged by the EDTA, and lessen or eliminate the decrease in the measured amount of extracellular vanadate. These findings demonstrate that the blood cells are accumulating vanadate against a concentration gradient, and that [^{48}V]vanadate influx is an accurate measure of this accumulation. Assuming that vanadium partitions into the total intracellular space of the vanadocytes, the amount of vanadium taken up in Fig. 1a raised the intracellular concentration by 2.5 mM. This net uptake therefore produces a 10-fold concentration ratio between the cell and the external solution.

Effect of metabolic inhibitors on influx

To determine whether the substantial accumulation of vanadate by tunicate blood cells is an active process, the effects of several metabolic inhibitors on influx were studied. It was found that neither inhibitors of glycolysis (2-deoxyglucose (10.9 mM), sodium fluoride (0.413 mM)); nor uncouplers of oxidative phosphorylation (CCMP (10.9 mM) and dinitrophenol (0.386 mM)) had any significant effect upon the rate of [^{48}V]vanadate influx (Table II). Small decreases in the extent (but not the initial rate) of vanadate influx were produced by 2-deoxyglucose, and by glucose.

TABLE I

NET ACCUMULATION OF VANADIUM BY BLOOD CELLS

Suspensions of fresh blood cells were incubated in isotonic Hepes/NaCl buffer at pH 8.0 with a known concentration of vanadate at 20°C for 15 min. In one experiment, EDTA was added to the suspension after this incubation period. The cells were spun down and the concentration of vanadium in the supernatant measured by atomic emission spectroscopy.

	Extracellular vanadate concentration (mM)	
	Initial	After 15 min
9% cell suspension	0.50	0.22 ± 0.005
7% cell suspension	0.50	0.29 ± 0.005
7% cell suspension	1.00	0.62 ± 0.005
7% cell suspension + 5 mM EDTA	1.00	0.69 ± 0.005

TABLE II

[^{48}V]VANADATE INFLUX IN THE PRESENCE OF METABOLIC INHIBITORS, SULFATE AND CHROMATE

In the experiments 1–4 aliquots of an 11% blood cell suspension were incubated with the indicated reagent for 20 min at 15°C prior to being made 0.50 mM in [^{48}V]vanadate and influx was measured as described in experimental procedures. In experiments 5–9, an 8% blood cell suspension was used, and the initial concentration of [^{48}V]vanadate was 0.53 mM.

	Reagent	Initial rate (μM/min)	Final extracellular [^{48}V]vanadate concentration (mM)
1 +	0.50 mM [^{48}V]vanadate	205	0.22
2	plus 0.386 mM dinitrophenol	205	0.22
3	plus 0.194 mM chromate	200	0.21
4	plus 0.413 mM fluoride	200	0.22
5 +	0.53 mM [^{48}V]vanadate	67	0.34
6	plus 11 mM sulfate	72	0.34
7	plus 11 mM glucose	66	0.43
8	plus 11 mM deoxyglucose	64	0.43
9	plus satd. CCMP	52	0.30

Inhibition of uptake by other anions

Substrate specificity was investigated using three oxyanions of similar size and structure to vanadate: phosphate, chromate, and sulfate. Only phosphate produces significant inhibition of [^{48}V]vanadate influx (Fig. 3a). The $K_{1/2}$ of phosphate is 0.15 mM; the maximum inhibition is 73%. [^{32}P]Phosphate is rapidly taken up by the blood cells ($t_{1/2}$ = 18 s at 0°C), but it is not accumulated against a concentration gradient (Fig. 3b). [^{35}S]Sulfate is not taken up by the cells (Fig. 3b) and neither sulfate nor chromate inhibited [^{48}V]vanadate influx at concentrations up to 10.9 mM and 200 μM, respectively (Table II).

Inhibition specificity was investigated using 4,4′-diisothiocyano (DIDS) and 4,4′-dinitro (DNDS) derivatives of stilbene disulfonate. DNDS is known to be a potent competitive inhibitor of anion exchange across the human red cell membrane [20], and DIDS

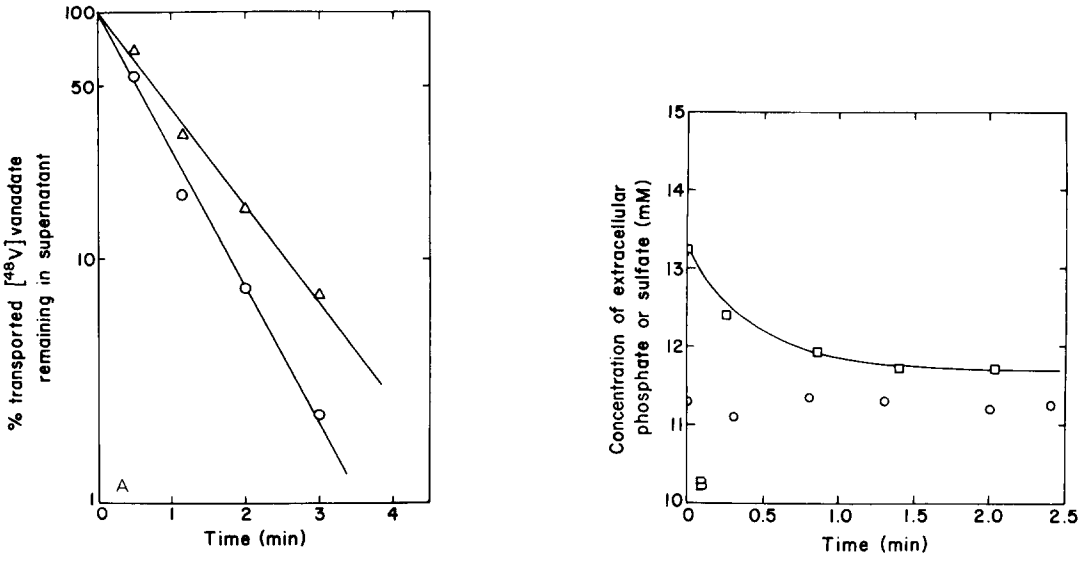


Fig. 3. (a) Inhibition of [^{48}V]vanadate influx by phosphate. One portion (△———△) of a 10% suspension of cells was made 155 μM in phosphate just prior to the addition of [^{48}V]vanadate to a concentration of 0.50 mM. The rate of uptake compared with that of the same cell suspension without the presence of phosphate showed 30% inhibition (○———○). (b) Uptake of [^{32}P]phosphate and [^{35}S]sulfate by unfractionated tunicate blood cells. □———□, a $1 \cdot 10^8$ cells/ml suspension was made 13.25 mM in [^{32}P]phosphate and maintained in an ice bath. Influx was measured as described and resulted in a final extracellular concentration of 11.71 mM [^{32}P]phosphate. A computer fit of the data gives $t_{1/2}$ = 20.5 s. ○, a 10% suspension of cells was made 13.8 mM in [^{35}S]sulfate and influx measured at 0°C. No influx was detected.

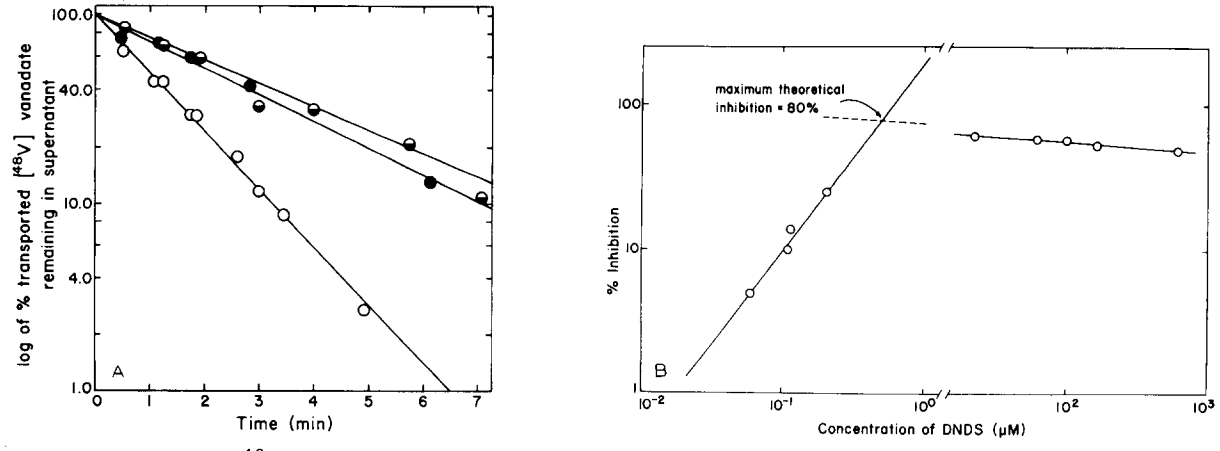


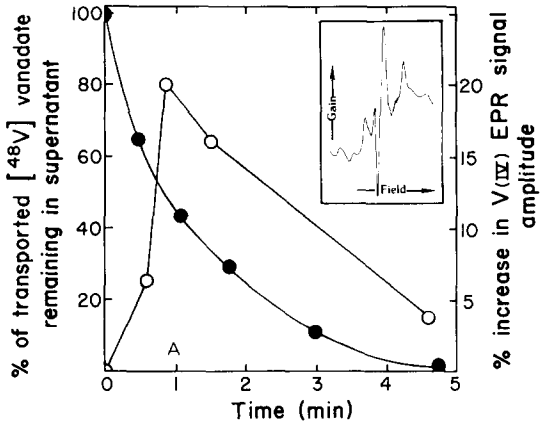
Fig. 4. (a) Inhibition of [^{48}V]vanadate influx into blood cells by DIDS and DNDS. A 10% cell suspension was made 0.46 mM in [^{48}V]vanadate, maintained at 0°C and influx measured as described. Uninhibited influx, ○———○ ($t_{1/2}$ = 0.96). Cells preincubated with 1.41 mM DIDS, ◒———◒ ($t_{1/2}$ = 2.45 min). Cells preincubated with 1.25 mM DNDS, ●———● ($t_{1/2}$ = 2.07 min). (b) Inhibition of [^{48}V]vanadate influx by DNDS. Varying concentrations of DNDS were added to 10% cell suspensions at room temperature and incubated for 20 min. The cell suspensions were then brought to 0°C, made 0.50 mM in [^{48}V]vanadate and the rate of influx measured.

reacts covalently with high specificity at the external transport site of the same system [21]. We now show that these inhibitors can also block [^{48}V]vanadate transport into vanadocytes (Fig. 4a). At low concentrations of DNDS the percent inhibition rises with increasing concentration (Fig. 4b). However, at high concentrations of DNDS, the percent inhibition falls with increasing concentration (Fig. 4b). Straight lines were drawn through the two sets (high and low concentration) of data, and their point of intersection taken to be the maximum theoretical inhibition of vanadate influx. The maximum experimental determined inhibition is 63% for DNDS; the theoretical value is 80%. The $K_{1/2}$ for inhibition is 260 nM; $K_{1/2}$ for the theoretical value is 300 nM.

Inhibition by DIDS was shown to be irreversible by extensively washing the DIDS-treated cells with medium containing 2% bovine serum albumin prior to measuring influx of vanadate. Therefore, since DIDS binds covalently, we report only the experimentally determined maximum percent inhibition of 61%. Experiments are now in progress using other derivatives of stilbene disulfonate to label and identify the plasma membrane components of the blood cells associated with anion transport. The fluorescent probe, eosin-5-maleimide, which specifically labels the anion exchange protein of human erythrocyte membranes [22], was found to compete with DIDS for binding sites on the cells. However, saturation of binding was not observed, and all vanadocyte membrane proteins detectable by Coomassie blue staining of a Laemmli gel [23] appear to react with the maleimide, as judged by fluorescence of the unstained gel. Therefore, labelling of vanadocytes by eosin-5-maleimide is nonspecific.

Fate of vanadate after uptake

Changes in the amount of reduced vanadium within the cells after addition of exogenous vanadate were monitored, to test whether the vanadocytes accumulate vanadate by reducing it to cationic species of V(IV) or V(III). The relative intracellular V(IV) concentration of frozen cell suspensions was determined by EPR spectroscopy. As can be seen from a plot of results (Fig. 5a), the EPR signal of the samples of the cell suspension increases over the period of vanadate influx into the cells, and subsequently decreases. This result supports the proposal that accumulation of vanadium is driven by reduction of anionic V(V) entering the cell. At pH greater than



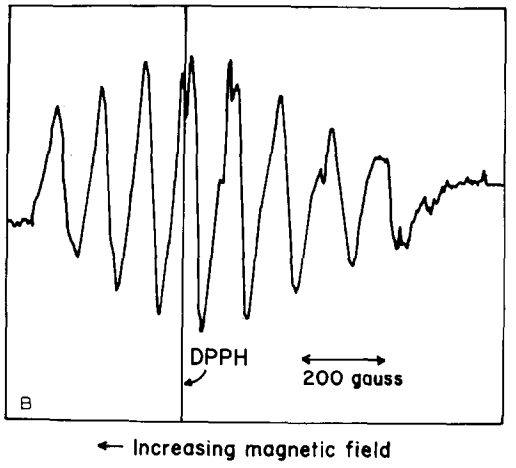


Fig. 5. (a) Increase in intracellular [V(IV)] during influx of V(V). o———o, a 10% cell suspension was divided into five aliquots which were each made 1 mM in V(V) and frozen at timed intervals. A room-temperature spectrum of the cell suspension gave the concentration of V(IV) at zero time. Insert: a spectrum of the frozen cell suspension; power = 8.0 MW, frequence = 9.3 GHz, modulation amplitude = 10, time constant = 1 s, gain = 12.5. •———•, a time course for [^{48}V]vanadate influx. (b) EPR spectrum of living blood cells. Washed cells were suspended in Hepes/NaCl buffer at a concentration of $6 \cdot 10^8$ cells/ml and loaded into a flat quartz cell. The spectrum was recorded at 9 MW microwave power, ν = 9550 GHz, time constant = 1 s, modulator amplitude = 106 and gain = $1.25 \cdot 10^3$. Broadening of all peaks and doubling of the two central peaks indicates the presence of two slightly different V(IV) species.

5.5, V(IV) forms an EPR-silent complex [19], so the V(IV) detected in these experiments cannot have been formed in the buffer (pH 8.0) by interactions between added V(V) and V(III) released by cell lysis, but must be an intracellular species.

The natural level of vanadyl present in the cells could be measured by EPR spectroscopy at room temperature (Fig. 5b). By comparison to a calibration curve relating peak height to the concentration of V(IV) in solutions of vanadyl sulfate in 1 M HCl [19], we have determined the concentration of V(IV) in the cells to be 5.7 mM. This value represents about 4% of the total vanadium in the cell [9]. This intracellular V(IV) concentration is similar to the value obtained for the V(IV) present in the vanadocytes of *Ascidia ceratodes* by Hodgson and coworkers, using X-ray absorption fine structure analysis (EXAFS) [11].

The room temperature EPR signal of the V(IV) within the cells is characteristic of a relatively low molecular weight vanadyl complex. If bound to a macromolecule, a vanadyl EPR signal characteristic of the frozen state would have been observed, since large molecules rotate slowly relative to the lifetime of the excited state of paramagnetic ions. Other reports on the state of vanadium in tunicate blood cells using NMR [10] and EXAFS [11] also suggest that intracellular vanadium is not bound to a protein or other macromolecule.

Discussion

Evidence has been presented for the existence of a specific vanadate transport system in the plasma membrane of blood cells from the tunicate *Ascidia nigra*. We have shown that vanadocytes are capable of a net accumulation of vanadate, which cannot be accounted for by nonspecific binding to the cell surface. Vanadate uptake by the vanadocyte is a highly specific process which can be saturated, and is inhibited by very low concentrations of the stilbene disulfonates, DIDS and DNDS. Surprisingly, neither of the stilbene disulfonates nor phosphate was found to block completely vanadate influx into tunicate blood cells. This observation may indicate that more than one process exists for vanadate influx. It is noteworthy, however, that certain inhibitors of anion transport in erythrocytes, such as eosin isothiocyanate, produce maximum inhibition of only 80%, even though a single anion exchange system is present in these cells [24].

Accumulation occurs against an apparent concentration gradient, but cannot be directly coupled to ATP hydrolysis. Neither uncouplers of oxidative phosphorylation nor sodium fluoride, an inhibitor of glycolysis, had any effect on vanadate influx. Both glucose and 2-deoxyglucose decreased the total amount of vanadate taken up by the cells, but not the rate at which influx occurs. A possible explanation of the decrease could be complex formation between the sugars and vanadate. *Cis*-diols are known to form complexes readily with vanadate [25].

Accumulation appears to be driven by the rapid reduction of intracellular vanadate to V(IV). Since the influx of [^{48}V]vanadate was observed to occur in a single exponential step, and to be paralleled by the appearance of V(IV) (as detected by EPR), it follows that the rate-limiting step of accumulation is the entry of vanadate into the cell. The possibility that extracellular vanadate is being reduced to V(IV) by V(III) from lysed cells is excluded since at pH 8.0 V(IV) exists only as an EPR-silent hydroxide [19]. Vanadocytes reduce V(V) to V(III) by two one-electron steps; if the reduction occurred in one two-electron step, no increase in the intracellular concentration of V(IV) would be observed. As the rate of influx slows, the amount of newly-generated V(IV) in the cells decreases almost to the pre-vanadate influx level. This decrease is very likely the result of further reduction to V(III), since no V(V), the product of oxidation, exists in the cells [1,2,10,11]. The electron donor for the reduction of V(V) to V(IV) has not yet been identified, but could be either tunichrome [12] or V(III). Both substances are present in high concentration within the cells, and both will reduce vanadate very rapidly to V(IV) ($t_{1/2}$ = 20 ms for the reduction of V(V) to V(IV) by tunichrome (Dingley, A.L., unpublished data)).

The observation that neither sulfate nor chromate interact with the vanadate transport system of tunicate blood cells indicates a high degree of substrate specificity. In contrast, the erythrocyte anion exchange system will accept a very broad spectrum of anions [26]. The basis of selectivity in the vanodocyte may be the negative charge on the ion, since all the oxyanions tested are tetrahedral and similar in

TABLE III

COMPARISON OF OXYANIONS

References are given in square brackets.

	M-O Bond length (Å) (M = V, P, Cr or S)	Species predominant at pH 8.0
H_3VO_4 [16]	1.66	$HVO_4^{2-}/H_2VO_4^- = 0.59$
H_3PO_4 [29]	1.52, 1.58, 1.57, 1.57	$HPO_4^{2-}/H_2PO_4^- = 7.9$
H_2CrO_4	1.65 [30]	$CrO_4^{2-}/HCrO_4^- = 100$ [31]
H_2SO_4 [32]	1.42, 1.43 1.52, 1.55	SO_4^{2-}

size (Table III). While sulfate and chromate are both divalent ions, significant proportions of the two transportable anions, vanadate and phosphate, are monovalent at pH 8.0. Vanadate has been shown to be a good phosphate analog for many enzymes [27]. In the proposed mechanism [12], it was suggested that sulfate acts as a counterion to the protons consumed in reducing vanadate to V(III), the sulfate being transported into the cells by the same system that transports vanadate. Transport into the cells as a counterion would also explain the low concentration of sulfate found in the blood plasma [28]. The present study does not support the proposed role of sulfate, but does not rule out that other anions may be involved.

Several characteristics of vanadate uptake by vanadocytes are similar to those observed previously for uptake by human erythrocytes [4,5]. Vanadate influx by erythrocytes is inhibited by DNDS, and is mediated by the anion transport channel, the band 3 protein [3]. However, influx into erythrocytes is biphasic: a fast step, which represents equilibration of vanadate across the cell membrane, is followed by a second slower step involving accumulation of vanadium as a result of intracellular reduction of transported V(V) to V(IV) [4,5]. The half-life for the first step is 4 min [4,5], which is much longer than the 20 s half-life of vanadate influx into vanadocytes. Whether this indicates a more efficient anion transport system in tunicate blood cells, or simply a larger number of transport sites, is unknown. The K_m for vanadate equilibration across the red cell membrane is 40 mM, an order of magnitude larger than that of vanadocytes, where $K_m = 1.4$ mM. The rate-limiting step for vanadium accumulation in erythrocytes is reduction of V(V) to cationic V(IV) by intracellular glutathione, whereas in the vanadocyte, entry of vanadate is rate-limiting. Moreover, the reduced vanadium within the erythrocyte is complexed by hemoglobin, whereas it appears to be unassociated with protein in vanadocytes.

Our experimental results explain some of the features of the tunicate blood cell; namely, why only vanadate is sequestered and why it is present in reduced form. Only vanadate and phosphate can enter the cell by the anion transport system and of the two, only vanadate forms a cation upon reduction. Thus, only vanadate can undergo the two-fold process required for accumulation: transport into the cell, followed by reduction to a non-transportable cation. Still unanswered are the questions of the identity of the intracellular reducing agent(s) which provide the driving force for accumulation of vanadium; whether vanadate influx is coupled to an efflux of other anions from the cell; and, the function of vanadium in the tunicate's physiology.

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