Isolation, Properties and Structural Studies on a Compound from Tunicate Blood Cells That May Be Involved in Vanadium Accumulation

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A novel compound, for which the trivial name tunichrome is proposed, was isolated from the vanadium-rich blood cells of the tunicate *Ascidia nigra*. Preliminary structural studies suggest a molecular weight of about 390, the presence of conjugated vinyl groups, and an acidic group, possibly carboxyl, with an apparent pK_a of 3.0. Elements C, H, N and O comprise 98.4% of the sample weight, the number of atoms per mol of tunichrome being 14.1, 22.2, 1.5 and 10.6 respectively, which indicates some heterogeneity in the sample. Tunichrome readily reduces Fe(III) and V(V). In an initial fast step, 2 mol of V(V) are reduced, or 4 mol of Fe(III)–phenanthroline per mol of tunichrome; in a further slow reaction, another 9 mol of Fe(III)–phenanthroline or Fe(III)–bipyridine are reduced. The initial reaction is first-order with respect to tunichrome and Fe(III). Above pH 3.5, tunichrome is rapidly hydrolysed, 13 mol of OH^- being consumed per mol of tunichrome. The hydrolysis involves polymerization and loss of the characteristic absorption peak at 325 nm. It is suggested that the presence of tunichrome may be linked to vanadium accumulation by the blood cells. The mechanism involves entry of vanadate via an anionic channel into vacuoles of the blood cells, where it is reduced to V(IV) or V(III), both of which, being cationic, cannot escape from the vacuole.

It has been known for several years that vanadium is an essential trace element in plants and animals (Underwood, 1971; Hopkins, 1976). Recently, it was shown to be a potent inhibitor of the $(Na^+ + K^+) - ATPase$ (Cantley *et al.*, 1977), to which it binds from the cytoplasmic side of plasma membranes and is accumulated by erythrocytes via the anion-exchange system (Cantley *et al.*, 1978). In most organisms, the vanadium is present in trace amounts. However, it is accumulated by certain species of tunicate to extraordinarily high concentrations, within a type of blood cell called the vanadocyte (Webb, 1956). It is stored in acidic vacuoles, as either V(IV) (the Aplousobranchia) or as a mixture of V(IV) and V(III) (the Phlebobranchia) (Rezaeva, 1964; Swinehart *et al.*, 1974). This type of vacuole, and hence the blood as a whole, often appears green–yellow, a colour produced not by the vanadium as was once believed (Bielig *et al.*, 1966; Goodbody, 1974), but by an organic chromogen (Swinehart *et al.*, 1974; Gilbert *et al.*, 1977; Macara *et al.*, 1979) for which we propose the trivial name tunichrome. Tunichrome is readily oxidized by molecular O_2 , Fe(III) and V(V) (Swinehart *et al.*, 1974; Macara *et al.*, 1979). Its function, and that of the vanadium, remain obscure. We have demonstrated, however, that the vanadium, does not func-

tion as an O_2 carrier, and that the vanadium–protein complex, haemovanadin, once thought to be the functional form of the vanadium *in vivo* (Bielig *et al.*, 1966; Goodbody, 1974), does not exist (Gilbert *et al.*, 1977).

In the present paper, preliminary structural studies on the chromogen and an examination of its reactions with Fe(III) and vanadium are presented, in an attempt to elucidate the role of tunichrome in the accumulation and metabolism of vanadium by the tunicate *Ascidia nigra*.

Materials and Methods

Specimens of *Ascidia nigra* were obtained from the coast of Florida and maintained as described previously (Gilbert *et al.*, 1977). Sephadex LH-20, G-10 and G-25 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.; 2,2'-bipyridine and 1,10-phenanthroline were from Sigma Chemical Co., St. Louis, MO, U.S.A.; $[^2H_6]$ dimethyl sulphoxide containing 1% (v/v) tetramethylsilane reference standard was from MSD Isotopes (Merck and Co., Rahway, NJ, U.S.A.); all other chemicals were from Fisher Scientific Co., Medford, MA, U.S.A. The Fe(III)–phenanthroline complex, Fephen $_3^{3+}$, was

prepared by oxidation of Fephen₃²⁺ with PbO₂ (Pelizzetti *et al.*, 1976). Vanadium(III) was prepared by electrolytic reduction of 0.05M-VOSO₄ in 1M-H₂SO₄, under argon, with the use of a mercury cathode and platinum anode, and stored under argon until used.

Purification of tunichrome

Blood was removed and extracted with 0.1M-HCl as previously described (Macara *et al.*, 1979). To raise the pH above 2, 0.5M-sodium citrate, pH2.4, was added, and the extract was loaded on to a column (2.5cm×10cm) of Sephadex LH-20. It was eluted with either a continuous or discontinuous methanol/water gradient, from 0 to 100% methanol. Typically, on preparative runs, 1 column vol. of each of 0, 25, 50 and 75% methanol in water (v/v) was passed through the column, followed by 100% methanol, the tunichrome-containing fractions being pooled and dried under reduced pressure. To minimize formation of inhomogeneities in the column, produced by shrinkage of the gel, the gel was packed after swelling in methanol and maintained compressed between column spacers. Iron and vanadium were determined as described previously (Macara *et al.*, 1979). Methanol concentrations were estimated from the refractive index of the eluent.

To determine yields and tunichrome concentration in the blood, known volumes of blood from five specimens were centrifuged (2000g×5min), the wet cell masses measured and the tunichrome concentrations determined after extraction in 0.1M-HCl by measurement of A₃₂₅. The extracts were then pooled in two groups and fractionated on Sephadex LH-20. Those fractions containing tunichrome were pooled, dried and weighed.

Purity was checked by further passage through Sephadex LH-20, eluted with methanol/chloroform (2:1, v/v), and by t.l.c. on silica gel (Brinkmann silica gel G, New York, NY, U.S.A.). Methanol, methanol/methyl acetate (1:1, v/v) and methanol/acetonitrile (1:1, v/v) were used as solvents, and the plates were developed with iodine vapour.

Structural studies

The molecular weight was estimated by filtration of tunichrome through a Pellicon PS filter (Millipore Corp., Bedford, MA, U.S.A.), with a nominal molecular-weight limit of 10³, and by freezing-point depression of aqueous solutions of tunichrome over a range of concentrations (4–18mg/ml), by using an Osmette 2007 precision osmometer (Precision Systems). The mean of three estimations was taken for each standard and sample.

Dried samples of tunichrome were heated in a melting-point apparatus to estimate thermal stability.

Absorption coefficients were determined on three

separately purified samples of tunichrome dissolved in 0.1M-HCl.

Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN, U.S.A.) for C, H, O and N, after drying for 10h at –10°C under reduced pressure.

Fourier-transform n.m.r. spectra were taken of solutions of tunichrome (approx. 20mg/ml) in [²H₆]-dimethyl sulphoxide on a Bruker-WH90 spectrometer; 100 scans were accumulated for each sample.

I.r. spectra of tunichrome in both mineral oil mulls and KBr pellets were taken on a Perkin-Elmer 567 grating infrared spectrophotometer.

Titration, hydrolysis and reactions with iron and vanadium

Aqueous solutions of tunichrome (1–2mg/ml) were titrated by addition of standard NaOH or HCl. Titrations were performed at 20°C. N₂ was bubbled continuously through the solutions to exclude CO₂. The absorbance at 660nm was determined after each addition of alkali or acid. Alkaline hydrolysis was performed in a similar manner to the titration, the pH being raised to a final value of approx. 10.0. Hydrolysis products were fractionated on columns of Sephadex G-10 (1cm×28cm) and G-25 (1.5cm×47cm), by using 0.1M-potassium phosphate, pH7.5, as eluent.

Stoichiometry of reaction with Fe(III) was investigated in several ways. (a) Tunichrome was added (0–0.23mg/ml final concentration) to 2mM-Fe(III) in 0.1M-HCl that had been purged with N₂. The tubes were sealed and kept dark for 18h, after which excess bipyridine was added. The Fe(II) was determined as its complex at 520nm, and at subsequent intervals up to 625h. In other experiments tunichrome was added to mixtures of Fe(III) and excess bipyridine in 0.1M-HCl, and the increase in absorbance at 520nm recorded. (b) The reaction between tunichrome (0–10μg) and Fephen₃³⁺ (2.5nM) in 0.1M-HCl was followed by determination of the formation of the reduced iron complex at 510nm. The Fephen₃³⁺ was prepared immediately before use and kept dark and on ice. Corrections were applied for photoreduction during the course of the reaction.

Initial rates of reaction of tunichrome (0–25μg/ml) with Fe(III) (0–50μM) in 0.1M-HCl were determined from the decrease in tunichrome absorbance at 325nm. Initial rates were determined by drawing tangents at time zero. All experiments were at room temperature (about 20°C).

The stoichiometry of the reaction of V(V) (0–50μM) with tunichrome (25μg/ml) in 0.1M-HCl or 1M-H₂SO₄ was also determined from the decrease in A₃₂₅. Solutions of higher concentration (0.05M-vanadium) in 0.1M-HCl or 1M-H₂SO₄ were also used, and spectra of the reaction products were recorded

for the visible region, to determine the final oxidation state of the vanadium. Similar experiments were performed by using V(IV) as substrate, either in large excess (0.05 M) or at a lower molar concentration than that of the tunichrome (5.0 mg of tunichrome/ml, 2.5 mM-vanadium).

Results

Purification of tunichrome

The use of Sephadex G-100 in the purification of tunichrome, described previously (Macara *et al.*, 1979), proved unsatisfactory on a preparative scale because repeated elutions damaged the gel, owing apparently to reaction of the chromogen with the dextran matrix. Sephadex LH-20 appears to be relatively inert, however, and allows control of separation, since tunichrome adsorbs to the material in water, but is eluted by methanol (Fig. 1). Iron and vanadium were eluted by water at the bed volume, V_t . The vanadium was visible as a pale-blue band on the gel, with an absorbance maximum at 760 nm, characteristic of V(IV). The three overlapping peaks which represent tunichrome (Fig. 1) could not be further resolved under the conditions used. Samples from each gave very similar visible-u.v. spectra, and reacted in the same way with Fe(III). Those fractions

containing the central peak were pooled and used in further investigations. On a further passage through Sephadex LH-20 with methanol/chloroform (2:1, v/v) as eluent, the tunichrome emerged as a single peak at V_t . On t.l.c., tunichrome invariably showed tailing and did not give a sharply defined band. However, no other bands were visible. Approximate R_F values were: for methanol, 0.67; for methanol/methyl acetate and methanol/acetonitrile, 0.45.

Structure and properties

The visible-u.v. spectrum of tunichrome fractionated on Sephadex LH-20 was identical with that described previously for tunichrome fractionated on Sephadex G-100 (Macara *et al.*, 1979), with a peak at 325 nm. The solid proved insoluble in apolar solvents such as benzene and chloroform. Although thermostable for short periods (Macara *et al.*, 1979), it is slowly hydrolysed (and oxidized by molecular O_2), even at -10°C . It was therefore stored under liquid N_2 .

Some 55% of the chromogen passed through the Pellicon PS filter, indicating a molecular weight of less than 10^3 , and possibly, assuming no adsorption and a spherical molecule, of around 500. Depression of freezing point was proportional to tunichrome concentration over the range 4–18 mg/ml and corres-

Fig. 1. Fractionation of *A. nigra* blood-cell extract on Sephadex LH-20

About 1 column vol. of each of 0, 25, 50 and 75% (v/v) methanol in distilled water was passed through the column, followed by 100% methanol. Fractions of about 5.0 ml volume were collected automatically. Absorbances were determined at 250 nm (●) and 325 nm (○). V_0 , void volume; V_t , bed volume. V and Fe (not shown) were eluted at V_t . Methanol concentration in the eluent (---) was determined from the refractive index.

Fig. 2. ^1H n.m.r. spectrum of tunichrome (approx. 20 mg/ml) in $[\text{}^2\text{H}_6]$ dimethyl sulphoxide containing 1% (v/v) tetramethylsilane reference standard

Spectrum was obtained at 90 MHz by Fourier-transform analysis of 100 scans over a sweep-width of 1200 kHz. The large peak at about 3.5 p.p.m. is due to water.

ponded to a number-average molecular weight of 388 ± 8 (± 1 s.e.; number of samples, $n = 24$).

The absorption coefficient at 325 nm, in 0.1 M-HCl, is 27.3 ± 0.35 litre $\cdot$ g $^{-1}$ $\cdot$ cm $^{-1}$, or, assuming the molecular weight to be 388, 10650 ± 410 M $^{-1}$ $\cdot$ cm $^{-1}$ ($n = 3$). Between pH values of 2.5 and 3.5 tunichrome solutions appear green, owing to the appearance of a broad absorption band in the visible region with a maximum at approx. 660 nm. The absorption coefficient at 660 nm is 0.13 ± 0.021 litre $\cdot$ g $^{-1}$ $\cdot$ cm $^{-1}$ or 50.4 ± 9 M $^{-1}$ $\cdot$ cm $^{-1}$ ($n = 3$).

The mean tunichrome concentration within the blood of *A. nigra* was 1.17 ± 0.26 mg/ml ($n = 5$). Cell volume, determined from wet-cell weights, was $2.3 \pm 0.4\%$ (percentage of blood volume). According to Vallee's differential blood count for *A. nigra* (Vallee, 1967), about 61% of the blood cells comprise vanadocytes. The tunichrome concentration within the vanadocyte is therefore of the order of 85 mg/ml or 0.2 M. Since tunichrome is located within the vacuoles of the vanadocyte (George, 1939), the intravacuolar concentration will be somewhat higher. A similar calculation, by using Carlson's (1975) data for vacuolar volume of vanadocytes from *Ascidia ceratodes*, gives an intravacuolar concentration of about 1.0 M.

Elemental analysis gave the following results (percentage by weight): C, 43.48%; H, 5.73%; N, 5.50%; O, 43.64%. These elements therefore constitute 98.35% of the sample weight. Assuming a

Fig. 3. Spectrophotometric acid-base titration of tunichrome at 660 nm

Tunichrome concentration was 1.8 mg/ml, in water. —, Forward titration with NaOH; ● and ■ represent different samples. ----, Back-titration with HCl. Absorbance values were corrected for dilution.

mol.wt. of 388, numbers of atoms per molecule will be: C, 14.1; H, 22.2; O, 10.6; N, 1.5.

Fig. 4. Fractionation of tunichrome hydrolysate on (a) Sephadex G-25 and (b) Sephadex G-10. The eluent in both cases was 0.2M-potassium phosphate, pH 7.5. V_0 , void volume; V_t , bed volume.

The n.m.r. spectrum (Fig. 2) shows bands corresponding to vinyl protons at 4.7, 6.3, 6.6 and 6.9 p.p.m. and broad bands at 8.1 and 8.8 p.p.m. and 9.97 and 10.1 p.p.m. The last may represent amide protons. Several other small peaks are visible, probably produced by contaminants. The only assignable peaks from the i.r. spectra were a vinyl $C=C$ stretching band at 1660cm^{-1} and a $C-O$ stretching band at 1026cm^{-1} . A broad $O-H$ stretching band was always present at 3300cm^{-1} , indicating either hydroxyl groups or a small amount of bound water, or both.

Titration and hydrolysis

Spectrophotometric titration of the chromophore is hindered by an irreversible hydrolysis that occurs above about pH 3.5. A plot of absorbance at 660nm against pH (Fig. 3) indicates, however, the presence of an ionizable group with an apparent pK_a of 3.0–3.1. The stoichiometry could not be reliably determined at the tunichrome concentrations used. The hysteresis observable upon back-titration with acid was probably a consequence of partial hydrolysis. Continued forward and reverse titrations caused a progressive increase in overall absorbance, owing to increased turbidity, and a decrease in the change around pH 3.0, as the amount of unhydrolysed tunichrome decreased.

Above pH 4.0 the solution rapidly turned yellow-brown, and the peak at 325 nm was lost. Reaction with

NaOH was initially rapid, but continued slowly for up to at least 100h. The equivalent weight of tunichrome for complete reaction appears to be 30 g/mol of OH^- added. Assuming a mol. wt. of 388, 13.0 ± 0.4 mol of OH^- are therefore consumed per mol of tunichrome.

Fig. 5. Stoichiometries of tunichrome reactions (a) Fast reaction with tunichrome over the range 3.1–8.5 mg of tunichrome/ml, in the presence of excess Fephen_3^{3+} , and 0.1 M-HCl. The line is the best fit to the points, found by linear regression. Assuming a tunichrome mol.wt. of 388 ± 8 , the slope gives a stoichiometry of 3.79 ± 0.27 mol of Fephen_3^{3+} reduced per mol of tunichrome. The intercept probably represents the extent of photoreduction of the complex in the absence of tunichrome. (b) Reaction of V(V) with tunichrome, estimated from the change in absorbance of tunichrome at 325 nm. Initial tunichrome concentration was $38\text{ }\mu\text{g/ml}$ in all cases, in 0.1 M-HCl (●) or 1 M- H_2SO_4 (○), and the reaction was started by the addition of 2–10 μl of V(V) in 0.1 M-HCl. The line is the best fit by linear regression to the data. The mol.wt. of tunichrome was assumed to be 388 ± 8 . The slope gives a stoichiometry of 2.16 ± 0.03 mol of V(V) reacting with 1 mol of tunichrome.

Fig. 6. Kinetics of reaction of Fe(III) in 0.1M-HCl with tunichrome, followed by determination of the decrease in absorbance of the tunichrome at 325 nm

Initial tunichrome concentration was 38 $\mu\text{g/ml}$ in all cases. Initial rates were estimated from drawn tangents, and converted into μM -tunichrome oxidized/min, assuming a mol.wt. for tunichrome of 388 ± 8 . The line is the best fit by linear regression to the data. Measurements were at room temperature (20°C). Overall rate constant is $31.5 \pm 3.2 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Hydrolysed solutions were initially slightly turbid; a small amount of acid-insoluble precipitate remained at the end of the reaction. The soluble hydrolysate product was too large to permeate the Sephadex G-10. It was eluted as a broad band on Sephadex G-25, indicating heterogeneity in size of the hydrolysis products (Fig. 4). No adsorption on the gels was observed.

Reaction with Fe(III)

Fe(III) in the presence of excess bipyridine was reduced by tunichrome in an initial fast reaction followed by a long slow reaction that was 90% complete after 120h. The equivalent weight of tunichrome, after complete reaction, was 30g/mol of Fe(III) reduced, which gives a stoichiometry of 13mol of Fe(III) reduced per mol of tunichrome.

The stoichiometry of the initial reaction was studied by using Fephen_3^{3+} because of the large difference in rates between the fast and slow steps with this reagent. The stoichiometry was constant over the range 4–85 $\mu\text{g/ml}$ (Fig. 5a) at a value of about 4mol of Fephen_3^{3+} reduced per mol of tunichrome.

Reduction of Fe(III) was accompanied by a proportional decrease in absorbance by the tunichrome at 325nm. This effect was utilized to follow the kinetics of reduction of Fe(III) in the absence of chelating agents. The initial reaction was first-order with respect to both iron (Fig. 6) and tunichrome (results not shown) concentrations. The overall rate constant was $31.5 \pm 3.2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (20°C , $I = 0.1, 0.1 \text{ M-H}^+$).

Reaction with vanadium

On addition of V(V) to tunichrome in acid solution, a transient red-brown colour was observed, concomitant with a decrease in the tunichrome absorbance at 325nm, and the appearance of a peak at 760nm, characteristic of the aquo-V(IV) vanadyl ion. The reaction with V(V) was too rapid to be followed by static methods. The stoichiometry of reaction, determined from the decrease in absorbance at 325 nm, was constant over the range 10–50 $\mu\text{M-V(V)}$ (Fig. 5b), at a value of 2.16 ± 0.03 mol of V(V) per mol of tunichrome. No reaction was observed under similar conditions when V(IV) or V(III) replaced V(V). In the presence of neither excess tunichrome nor excess V(IV) was any change in the absorbance of the vanadyl ion at 760nm detectable. The low absorption coefficient of the aquo-vanadyl ion ($17 \text{ M}^{-1} \cdot \text{cm}^{-1}$) precluded detection with certainty of any small change in V(IV) concentration, however [less than approx. 10% of total V(IV) concentration]. Fractions eluted from the Sephadex LH-20 column at the void volume and bed volume gave similarly negative results, when tested for their ability to reduce V(IV).

Discussion

In the vanadocytes of *A. nigra*, tunichrome occurs within the same vacuoles as those containing vanadium. The intravacuolar concentration of the metal, which is probably of the order of 1.0M (Carlson, 1975), is a value close to that calculated in this report for the concentration of tunichrome. Moreover, there is a surprisingly small variation in the concentrations of both vanadium (Carlson, 1975) and tunichrome ($1.17 \pm 0.26 \text{ mg/ml}$ of blood) between individual specimens, which suggests tight control of their metabolism. These facts make reasonable a linkage of function between vanadium and tunichrome, and we have suggested previously (Macara *et al.*, 1979) that the purpose of the chromogen is to reduce vanadium from the 5+ oxidation state, in which it is present in sea water, to the 3+ oxidation state which appears to predominate within the vanadocytes of the Ascidaceae (Boeri & Ehrenberg, 1954; Rezaeva, 1964; Swinehart *et al.*, 1974).

The evidence presented here demonstrates that tunichrome can readily reduce vanadium to V(IV). Further reduction was not detectable under the conditions used, however. Another rapidly reacting labile electron-transfer compound in the vacuoles may be responsible for this step, although it remains possible that tunichrome itself will reduce the V(V) to V(III) over an extended period, if present in sufficiently high concentration. Ascorbic acid is known to reduce V(V) to V(IV) in a fast reaction (Kustin & Toppen, 1972), followed by slower steps that eventually result in V(II) (Kahn & Martell, 1968). The mechanism of

reduction both by ascorbate and by catechols (Kustin *et al.*, 1974) appears similar to the mechanism of reduction by tunichrome in that a transient red- or brown-coloured intermediate is formed, which has been identified in these other systems as a complex of V(V) with the reducing agent.

Whether or not tunichrome is the compound involved, reduction of vanadium to either V(IV) or V(III) allows the operation of a simple mechanism for its accumulation to high concentrations. Vanadium exists in sea water in its 5+ oxidation state, as the anion vanadate (McLeod *et al.*, 1975), and in the blood-cell vacuole as either V(III) or V(IV). Orthodox mechanisms, in which vanadium is coupled by either symport (Fig. 7a) or antiport (Fig. 7b) to a proton pump in the vacuolar membrane, are inadequate if considered in isolation from the reduction process. Symport, which would in fact be formally equivalent to transport of the neutral species H_3VO_4 , requires the proton gradient to be the reverse of that observed (Webb, 1956; Bieligi *et al.*, 1966). Antiport, following cytoplasmic reduction to V(IV), would on the other hand require an extraordinarily large ΔpH across the vacuolar membrane, and although it cannot be ruled

out, must be considered unlikely. Early evidence that the vacuoles contain 0.5–1 M H_2SO_4 (Webb, 1956) is suspect in that it was based upon titration of lysed cells with NaOH, which would cause the hydrolysis and oxidation of the V(III) from them, resulting in a sizeable overestimation of the initial proton concentration. Moreover, maintenance of a large ΔpH would entail an energy expenditure probably inconsistent with the observation (Deck *et al.*, 1966) that native vanadocytes possess few, if any, mitochondria.

An alternative mechanism is shown in Fig. 8. Accumulation of vanadium is passive in the sense that vanadate diffuses into the vacuole down a concentration gradient, since upon entry it is immediately converted into the cationic dioxovanadium species, VO_2^+ , and is reduced to either the 4+ oxida-

Fig. 7. Possible mechanisms for vanadium accumulation by vanadocyte vacuoles, involving coupling to a proton pump (p) (a) Symport of vanadate V(V). (b) Antiport of vanadyl V(IV). It is assumed that vanadium is transported from the plasma into the cytoplasm as vanadate. No mechanisms involving cytoplasmic reduction to V(III) are permissible, given the instability of this species at physiological pH. v is vanadium porter.

Fig. 8. Model for control of vanadium accumulation by reduction of V(V)

Vanadate diffuses into the vacuole through an anion channel, or via an anion/proton uniporter (v), which is specific for oxyanions. In the acidic environment of the vacuole, the vanadate is reduced to V(IV) or V(III). Sulphate enters as counter ion to the protons consumed during reduction. The vacuole membrane is assumed to be impermeable to cations. Equations show steps in reduction of V(V) to V(III) in acid solution.

tion state, VO^{2+} , or the 3+ oxidation state, V^{3+} . The extent of accumulation is controlled by the concentration of reductant. Sulphate would act as the counter ion. Both could enter the vacuole through an anionic channel, or alternatively as the formally neutral species H_3VO_4 and H_2SO_4 , via a proton/anion uniporter. The latter mechanism has been suggested by Mitchell & Moyle (1969) for uptake of phosphate and sulphate by rat liver mitochondria. As Mitchell (1970) has pointed out, the very low concentrations at which these neutral species exist at physiological pH are irrelevant, since they would be formed catalytically at the translocator centre of the specific porter. Alternatively, the vanadate and protons could be carried at separate sites on the porter. Vanadate uptake by human blood cells has been shown to occur via the plasma-membrane anion-exchange system (Cantley *et al.*, 1978).

It is noteworthy that vanadium will accumulate within any compartment whose walls are permeable to anions but not cations, and whose internal environment is acidic, even in the absence of a reductant, since V(V) is predominantly anionic above pH 3, and cationic below this pH. For instance, the concentration gradient, at equilibrium, across a suitable membrane separating sea water and a solution of pH 1.0 would be about 10^5 .

The proposed model explains several interesting observations on tunicate blood chemistry. Firstly, it has been found that the plasma of the Ascididae, which is iso-osmotic with sea water, is anomalously low in sulphate and carbonate, and high in chloride (Goodbody, 1974). This distribution is explicable if it is assumed that the vanadocyte porter is specific for oxyanions (such as sulphate, vanadate, carbonate or phosphate). Chloride could not then act as the counter ion in vanadium uptake and phosphate is present at too low a concentration in sea water to compete significantly. Secondly, the model explains the extraordinarily high specificity of the vanadocytes for vanadium. Other metal ions are present at only very low concentrations (Swinehart *et al.*, 1974). Only those species that exist as oxyanions in sea water and form cations upon reduction in an acidic environment will be accumulated by the proposed mechanism. Of all the elements present in sea water apart from vanadium, only chromium appears to behave in this manner. Since the concentration of chromium in sea water is around 1 nM (Horne, 1969), about 50-fold lower than that of vanadium, it would accumulate to a much smaller extent, even if no other selection process were involved.

The structure of tunicrome remains to be established. The n.m.r. spectrum indicates the presence of several vinyl groups, which, given the high absorption coefficient, should be conjugated. The elemental analysis indicates a large proportion of oxygen in the molecule, some of which may be present in one or

more carboxy groups. The apparent pK_a of 3.0 is consistent with the presence of a carboxy group, and is of some interest in that several previous workers (Boeri & Ehrenberg, 1954; Bielig *et al.*, 1966) have remarked upon the instability of blood-cell lysates above about pH 2.5, which they associated only with oxidation of V(III). Moreover, the brown colour of the lysate, termed Henze's solution (Bielig *et al.*, 1966), was assumed to be caused by either a vanadium-protein complex or a dimer of V(III) produced by hydrolysis (Kustin *et al.*, 1976), whereas it now seems more likely to be a result of the hydrolysis of tunicrome. The properties of the hydrolysate suggest that polymerization of the tunicrome occurs, above pH 3.5.

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