

⁵¹V-n.m.r. analysis of the binding of vanadium(V) oligoanions to sarcoplasmic reticulum

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(Received 19 April 1985; accepted 28 May 1985)

The binding of mono- and oligo-vanadates to sarcoplasmic reticulum was analysed by ⁵¹V-n.m.r. spectroscopy. The observations indicate that, in addition to monovanadate, the di-, tetra- and deca-vanadates are also bound to sarcoplasmic-reticulum membranes with high affinity. The binding of the vanadate oligoanions may explain some of the effects of vanadates on the conformation and crystallization of Ca²⁺-transport ATPase.

The inhibition of the Ca²⁺-transport ATPase of sarcoplasmic reticulum by vanadate (Pick, 1982; Pick & Karlsh, 1982) is usually explained by the tight binding of orthovanadate to the enzyme as an orthophosphate or a transition-state analogue. This explanation is supported by striking similarities in the chemistry of vanadates(V) and phosphates (Chasteen, 1983). The V–O bond length of vanadates is approx. 0.17 nm, compared with 0.152 nm for the P–O bond in orthophosphate. The slightly weaker acidity of H₃VO₄ (pK_a approx. 3.5, 7.8 and 12.5) as compared with H₃PO₄ (pK_a approx. 1.7, 6.5 and 12.1) may contribute to the higher affinity of vanadate, as compared with phosphate, for the enzyme (Pope *et al.*, 1980). Vanadate may also acquire a stable trigonal bipyramid geometry resembling the proposed transition-state structure of phosphate (Pope & Dale, 1968; Lindquist *et al.*, 1973), although the properties of enzyme-bound vanadate are as yet unknown.

The inhibition of ATPase activity by the formation of a kinetically stable E₂–VO₄ intermediate may be only one of several possible modes of actions of vanadate, since there are indications for the interaction of vanadate oligoanions with the Ca²⁺-transport ATPase. For example, the rate of crystallization of Ca²⁺-transport ATPase is markedly enhanced by increasing the vanadate concentration of the crystallization medium to 5 mM (Dux & Martonosi, 1983a,b; Varga *et al.*,

1985), where vanadate(V) oligomers accumulate (Boyd & Kustin, 1985). Decavanadate (V₁₀O₂₈⁶⁻), an octahedrally co-ordinated vanadate(V) polymer, forms tight complexes with the Ca²⁺-transport ATPase, and is particularly effective in promoting the formation of crystals of Ca²⁺-transport ATPase at low vanadate concentration (Csermely *et al.*, 1985).

We utilized the superb resolving power of ⁵¹V n.m.r. (O'Donnell & Pope, 1976; Howarth & Jarrold, 1978; Habayeb & Hileman, 1980; Heath & Howarth, 1981; Rehder, 1982; Domaille, 1984) to characterize the interaction of mono- and oligo-vanadates with the sarcoplasmic reticulum. The following aspects were investigated: (1) the composition of monovanadate and decavanadate solutions with respect to the relative concentrations of the various vanadate(V) polyanions; (2) the influence of conditions known to affect the interaction of vanadate with the Ca²⁺-transport ATPase on the equilibrium between the various anionic forms of vanadate; (3) the binding of mono-, di-, tetra- and deca-vanadates to the sarcoplasmic reticulum and competition between the different vanadate species for the binding sites.

The results suggest that tight binding of tetra- and deca-vanadates to the Ca²⁺-transport ATPase may be involved in the stabilization of the conformation required for the formation of membrane crystals of Ca²⁺-transport ATPase.

Experimental

⁵¹V-n.m.r. measurements

N.m.r. measurements were made at a vanadium frequency of 94.69 MHz on a Bruker WM 360WB

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spectrometer interfaced with an Aspect 2000 computer, with a pulse Fourier-transform n.m.r. technique (90° pulses). The chemical-shift values are given with reference to VOCl_3 as a standard. Typical spectral widths of 42 kHz in 4096 data memory were used. Before Fourier transformation of the spectra, 10–30 Hz exponential line broadening was applied. Spectra were recorded with 10 mm-pathlength samples in 2.5 ml total volume of the standard buffer of 0.1 M-KCl/5 mM- MgCl_2 /0.5 mM-EGTA/10 mM-imidazole, pH 7.4, usually at 25°C, but for long runs at 2°C. Spectra were generally obtained from the accumulation of 4000 transients at ten scans/s; thus the samples were at room temperature for only 10–15 min.

Preparation of 'monovanadate' and 'decavanadate' solutions

Stock solutions of monovanadate (50 mM) were prepared by boiling freshly made aqueous solutions of Na_3VO_4 at pH 10 for 15 min. Decavanadate solutions were prepared by adjusting the pH of a 50 mM- Na_3VO_4 solution to 4.0, and after some hours re-adjusting the pH to 7.4. Under these conditions the decavanadate is a thermodynamically unstable but kinetically inert species in the solution. Its decomposition rate to oligo- and mono-vanadate at neutral pH is significantly faster in buffered solutions than in water, and sharply increases with temperature. Therefore care must be taken to use only freshly diluted ice-cold decavanadate solutions.

Determination of the decavanadate content of 'decavanadate' solutions by guanidine precipitation

We utilized the observation made by Soman *et al.* (1983) that decavanadate can be selectively precipitated by guanidine. To a set of samples containing decavanadate in increasing concentration (0.1–5 mM-vanadate) guanidine was added to a final concentration of 0.2 M. After 10 min in ice the precipitate was centrifuged, and the vanadate content of the supernatant was analysed by the method of Goodno (1979) as described previously (Varga *et al.*, 1985). The amount of vanadate precipitated was determined by comparing the vanadium concentration of the supernatant with the total concentration of vanadium in guanidine-free controls. The extent of decavanadate removal (usually over 80%) was checked by measurements of absorbance at 400 nm and by n.m.r. spectroscopy. The data are corrected for the amount of the remaining decavanadate.

Preparation of sarcoplasmic-reticulum vesicles

Sarcoplasmic-reticulum vesicles were prepared from predominantly white skeletal muscles of rabbits as described previously (Nakamura *et al.*,

1976). Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Preparation of soya-bean phosphatidylcholine liposomes

The chloroform/methanol stock solution of soya-bean phosphatidylcholine was dried under a stream of N_2 . The phosphatidylcholine was mixed with the standard buffer solution (0.1 M-KCl/5 mM- MgCl_2 /0.5 mM-EGTA/10 mM-imidazole, pH 7.4) and sonicated with a Heat Systems Ultrasonics W 185 sonifier at 95 W for 30 min in salted ice, under N_2 .

Materials

VOCl_3 (99.99% pure) was a product of Aldrich Chemical Co., Milwaukee, WI, U.S.A. Soya-bean phosphatidylcholine was obtained from Associated Concentrates, Woodside, NY, U.S.A. Ludox was a gift from E. J. DuPont de Nemours Co., Industrial Chemicals Department, Wilmington, DE, U.S.A. KCl, K_2HPO_4 and Na_3VO_4 were purchased from Fisher Scientific Co., Rochester, NY, U.S.A., and sodium tripolyphosphate was from La Pine Scientific Co., Irvington, NY, U.S.A. Arginine, histidine and lysine were obtained from Mann Research Laboratories, New York, NY, U.S.A. Guanidinium chloride was from Polysciences, Warrington, PA, U.S.A., and bovine serum albumin, EGTA, glycogen, imidazole, MgCl_2 , $\text{K}_4\text{P}_2\text{O}_7$ and 4-(2-pyridylazo)resorcinol were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Aqueous solutions of vanadium(V) form a complex system consisting of a dozen or more molecular species of vanadate (Baes & Mesmer, 1976; Choate & Mansour, 1979; Chasteen, 1983; Boyd & Kustin, 1985); their proportions are dependent on the pH, vanadate concentration, temperature and the ionic composition of the incubation medium. The principal components of the system (Fig. 1a) are the monovanadate anions (VO_4^{3-} , HVO_4^{2-} and H_2VO_4^-), vanadate dimers ($\text{HV}_2\text{O}_7^{4-}$ and $\text{H}_2\text{V}_2\text{O}_7^{3-}$), vanadate tetramers ($\text{V}_4\text{O}_{12}^{4-}$), vanadate pentamers ($\text{V}_5\text{O}_{15}^{5-}$) and vanadate hexamers ($\text{V}_6\text{O}_{17}^{4-}$ and $\text{V}_6\text{O}_{18}^{4-}$). At high vanadate concentration and moderately acidic pH decavanadates are the dominant species ($\text{V}_{10}\text{O}_{28}^{6-}$). The decavanadates are thermodynamically unstable at neutral pH and low vanadate concentration, but their rate of decomposition is so low that decavanadates may exist in significant concentration at pH 7.4 in standard solutions used for enzymic assays (Figs. 1b and 1c).

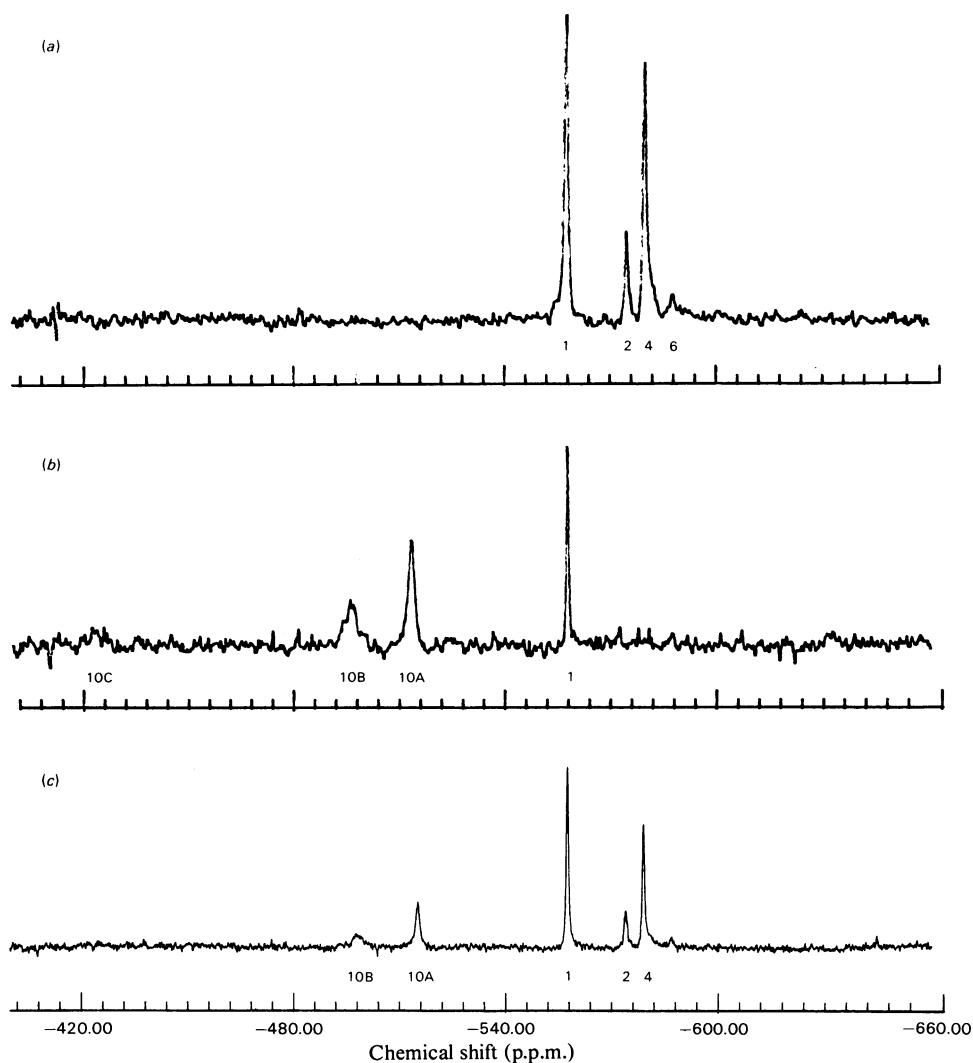


Fig. 1. Representative n.m.r. spectra of 'monovanadate' and 'decavanadate' solutions (a) Monovanadate (1 mM) in standard buffer solution (0.1 M-KCl/5 mM-MgCl₂/0.5 M-EGTA/10 mM-imidazole, pH 7.4) at 25°C. (b) Decavanadate (1 mM) in standard buffer solution as described in (a). (c) Mixture of monovanadate and decavanadate (1 mM each). The bands are designated as follows: 1, monovanadate; 2, divanadate; 4, tetra- vanadate; 6, hexavanadate (perhaps together with pentavanadate). 10A, 10B, and 10C are the three bands of decavanadate, related to the three environmentally distinct vanadate atoms (Howarth & Jarrold, 1978).

The distinctive n.m.r. spectra of the mono- and poly-anionic vanadate species permits their identification and analysis in complex mixtures.

Effect of vanadate concentrations on the composition of monovanadate and decavanadate solutions

In 0.1 M-KCl/5 mM-MgCl₂/0.5 mM-EGTA/10 mM-imidazole, pH 7.4, solutions at 25°C monovanadate is the dominant species at total vanadate concentrations of 0.2–0.5 mM (Fig. 2a). As the vanadate concentration is raised, di- and tetra- vanadates rapidly accumulate; at 10 mM-vanadate

concentration the equilibrium is strongly in favour of the tetra- vanadate, and monovanadate is present only in trace amounts (Fig. 2a).

Decavanadate stock solutions diluted into the standard medium give rise to three bands at -518 (10A), -495 (10B) and -423 p.p.m. (10C) that are characteristic of the three environmentally distinct vanadate atoms within the decavanadate molecule (Fig. 1). Additional components of the spectrum at -558, -570, -574 and -580 p.p.m., at vanadate concentrations greater than 2–3 mM, are attributable to mono-, di-, tetra- and hexa-meric vanadates

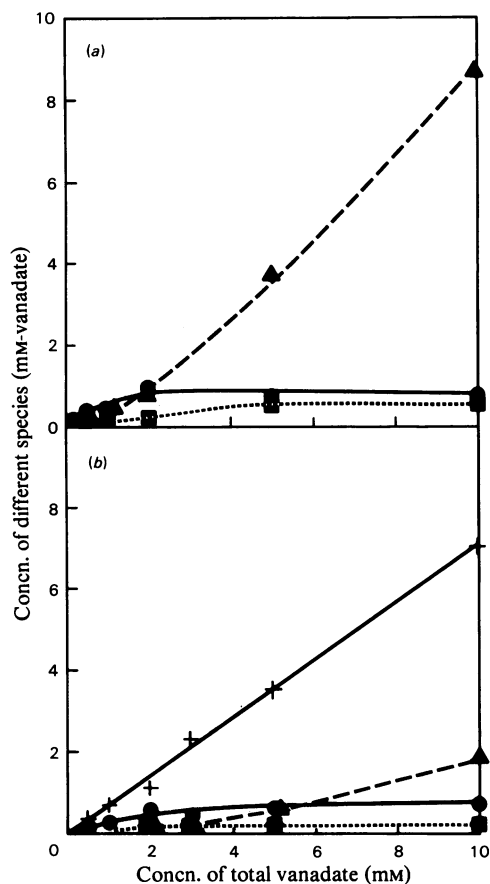


Fig. 2. Relative concentrations of the different ionic forms of vanadate in monovanadate and decavanadate solutions at various total vanadate concentrations

(a) Monovanadate solution; (b) decavanadate solution. Both solutions were prepared in 0.1M-KCl/5mM-MgCl₂/0.5mM-EGTA/10mM-imidazole, pH 7.4, at 25°C. The areas under the peaks were analysed and plotted against the total vanadate concentration. Symbols: ●, monovanadate; ■, vanadate dimers; ▲, vanadate tetramers; +, sum of the three peaks of decavanadate.

respectively. The relationship between total vanadate concentrations and the concentrations of the individual vanadate species in decavanadate solutions is numerically given in Fig. 2(b), on the basis of integration of the areas under the peaks. The values for decavanadate content of decavanadate solutions determined by n.m.r. and by guanidine precipitation were in reasonable agreement (Table 1).

Effect of pH on the composition of vanadate(V) solutions

Vanadic acid (H₃VO₄) has pK values of 3.5, 7.8 and 12.5. It is expected, therefore, that changes in

Table 1. Decavanadate content of freshly diluted decavanadate solutions

Decavanadate stock solution was diluted with standard buffer at 2°C to the indicated final vanadate concentrations, and the concentration of the decavanadate species was determined by n.m.r. and by guanidine precipitation as described in the Experimental section. The decavanadate content is expressed as percentage of the total vanadate concentration.

Concn. of total vanadate (μM)	Decavanadate content (%)	
	N.m.r.	Guanidine precipitation
500	70	64
1000	68	69
2000	77	71
5000	72	71

the extent of protonation over a wide pH range will affect the n.m.r. spectrum of vanadate solutions both in terms of the relative concentration of the various ionic species and their chemical shifts. Spectra of 1mM-vanadate solution were obtained in 0.1M-KCl/5mM-MgCl₂/0.5mM-EGTA/10mM-imidazole at 25°C at pH values ranging from 12 to 2.2 (not shown). With decreasing pH the amplitude of the monovanadate peak progressively decreased, and a large chemical shift occurred between pH 10 and 8.5 from -535 to -560 p.p.m. Significant contributions by di- and tetra-vanadates were observed at pH 8.3-6.5, and the broad peaks of decavanadate appeared at pH 4.1 and 2.2. The chemical shifts and the relative contributions of different vanadate species observed by us are close to those expected from the known pK values of the vanadate(V) anion (Howarth & Jarrold, 1978; Habayeb & Hileman, 1980; Heath & Howarth, 1981). In view of the large influence of pH on the equilibrium composition of vanadate solutions, all experiments were performed at pH 7.4.

Rate of conversion of decavanadate

'Decavanadate' stock solutions were diluted either in water or in KCl/imidazole buffer, at pH 7.4, and the rate of conversion of decavanadate into lower oligoanionic forms was followed by measurement of the disappearance of the orange-yellow colour of decavanadate at 400nm. The rate of decavanadate conversion at vanadate concentrations ranging from 0.05mM to 5mM, at 2°C, was rather low, with half-times of several hundred hours (Table 2). The activation energies are of the order of 94.1 kJ/mol (22.5 kcal/mol) in water and 97.1 kJ/mol (23.2 kcal/mol) in KCl/imidazole buffer. The decay rate of decavanadate was faster at 25°C than at 2°C, and was significantly greater in

Table 2. Rate of decavanadate decomposition

The half-times of decavanadate decomposition were determined by monitoring the change in the absorbance of the solutions at 400nm. The standard buffer solution was 0.1M-KCl/5mM-MgCl₂/0.5mM-EGTA/10mM-imidazole, pH7.4.

Solution	Half-time of decavanadate decomposition (h)	
	At 2°C	At 25°C
50 μM-Decavanadate in water	347.0	19.0
5mM-Decavanadate in water	2310.0	43.0
50 μM-Decavanadate in standard buffer	70.7	4.4
5mM-Decavanadate in standard buffer	101.9	4.5

KCl/imidazole buffer than in water at comparable pH values.

The changes in composition of the solution at 1mM-vanadate concentration during 24h of incubation at 25°C are numerically expressed in Fig. 3. During decavanadate decay there is an increase in the di- and tetra-vanadate concentration, whereas the concentration of monovanadate changes only slightly. In view of the significant decomposition of decavanadate in dilute solutions at room temperature, the experiments involving decavanadate were either performed at 2°C, or were limited to a few hours at 25°C.

Changes in the composition of vanadate solutions in the presence of sarcoplasmic-reticulum vesicles

The n.m.r. spectrum of a 2mM-vanadate solution in 0.1M-KCl/5mM-MgCl₂/0.5mM-EGTA/10mM-imidazole, pH7.4, is characterized by peaks representing the mono-, di- and tetra-vanadate species, with some hexavanadate in trace amounts. Sarcoplasmic-reticulum vesicles in concentrations ranging from 1 to 10mg of protein/ml selectively decreased the amplitude of the tetravanadate peak, suggesting preferential binding of tetravanadate, as compared with mono- or di-vanadate, to the sarcoplasmic-reticulum membrane (Fig. 4a).

In freshly diluted decavanadate solutions the three decavanadate resonances are dominant (Fig. 4b); the monomer contribution is relatively minor, but increases with incubation time, accompanied by the appearance of di- and tetra-vanadates. Sarcoplasmic-reticulum vesicles selectively diminish the amplitudes of the decavanadate peaks, with only slight effect on the monomer concentration, indicating preferential binding of decavanadate to the membrane (Fig. 4b). At a sarcoplasmic-reticulum concentration of 20mg/ml the decavanadate peaks were undetectable, even after overnight analysis (16h) that involved the averaging of more than half a million transients.

The preferential binding of vanadate decamers, tetramers and dimers to sarcoplasmic-reticulum

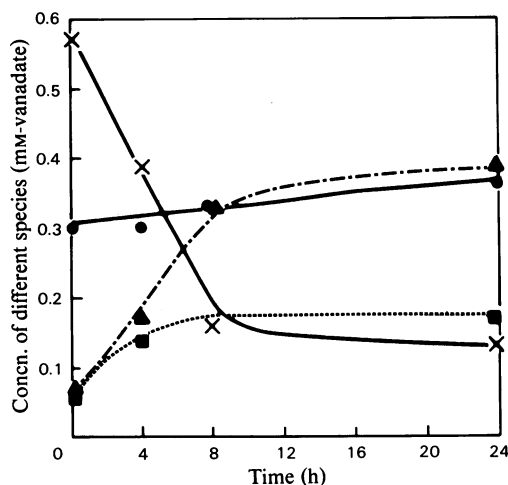


Fig. 3. Conversion rate of decavanadate at 25°C

A decavanadate stock solution was diluted in standard buffer containing 0.1M-KCl/5mM-MgCl₂/0.5mM-EGTA/10mM-imidazole, pH7.4, to final concentration of 1mM; the ⁵¹V-n.m.r. spectra were measured immediately and 4, 8 and 24h later at 25°C. The areas under the peaks were evaluated and plotted as the function of incubation time. Symbols: ●, monovanadate; ■, divanadate; ▲, tetra-vanadate; ×, decavanadate.

vesicles is particularly clearly observed by analysis of solutions containing mixtures of 1mM-mono-vanadate and 1mM-decavanadate (Figs. 4c and 5). Only a slight decrease in mono- and di-vanadate concentration follows the nearly complete disappearance of deca- and tetra-vanadate peaks from the spectrum, as the concentration of sarcoplasmic-reticulum protein is increased to 12mg/ml or above (Fig. 4c). The disappearance of di- and tetra-vanadate peaks upon addition of sarcoplasmic-reticulum vesicles can be attributed to the binding of these species to the membrane, since after ultracentrifugal sedimentation of microsomal

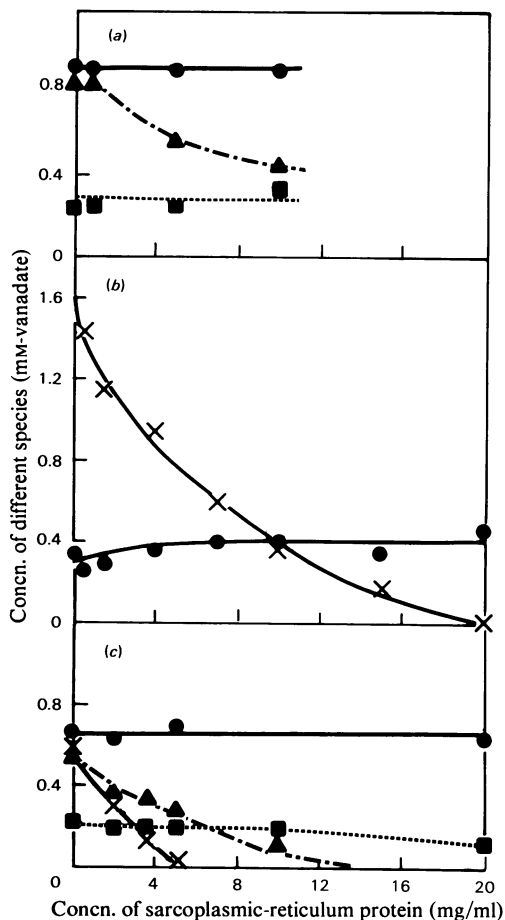


Fig. 4. Interaction of vanadate oligoanions with sarcoplasmic reticulum

(a) Monovanadate solution (2 mM); (b) decavanadate solution (2 mM); (c) mixture of 1 mM-monovanadate and 1 mM-decavanadate solution. All solutions were prepared in 0.1 M-KCl/5 mM-MgCl₂/0.5 mM-EGTA/10 mM-imidazole, pH 7.4. The areas under the peaks were evaluated and plotted as the function of the protein concentration (mg/ml) of sarcoplasmic reticulum. Symbols: ●, monovanadate; ■, divanadate, ▲, tetra- vanadate; ×, decavanadate.

particles neither the supernatant nor the sediment fractions reveal the presence of the di- and tetra- vanadate components. The disappearance of the decavanadate peaks in the presence of sarcoplasmic reticulum requires a more complex explanation, since in this case the microsome-free supernatant obtained after centrifugation contains a small, but significant, decavanadate signal. We assume that exchange between bound and free decavanadate may contribute to the broadening of the decavanadate signal.

Glycogen (10 mg/ml), Ludox particles (18 mg/ml) and suspension of single-walled liposomes (10 mg/ml), prepared by ultrasonic dispersion of soya-bean phospholipids in the standard medium, did not affect significantly the linewidths or the amplitudes of the peaks observable in 1 mM-'monovanadate' or 2 mM-'decavanadate' solutions. Therefore line broadening by physical heterogeneity of the vesicle suspensions is not expected to contribute significantly to the disappearance of tetra- and deca- vanadate signals in sarcoplasmic-reticulum suspensions.

Discussion

'Monovanadate' and 'decavanadate' solutions, which are commonly used as inhibitors in analysing the mechanism of phosphohydrolase and phosphotransferase enzymes, contain, in addition to monovanadate and decavanadate, several stable vanadate(V) oligoanions (di-, tetra- and hexavanadates), which are readily identified by ⁵¹V-n.m.r. spectroscopy. Vanadate(V) dimers, tetramers and decamers bind to sarcoplasmic-reticulum membranes with relatively high affinity, and are likely to contribute to the dramatic increase in the rate of crystallization of Ca²⁺-transport ATPase induced by dilute solutions of decavanadate, or by orthovanadate solutions at vanadate concentrations exceeding 1 mM (Dux & Martonosi, 1983a,b,c; Varga *et al.*, 1985).

Therefore, in addition to the formation of a kinetically stable E₂-VO₄ enzyme intermediate that is responsible for the inhibition of ATPase activity and Ca²⁺ transport (Pick, 1982; Pick & Karlsh, 1982), co-ordination of the highly charged di-, tetra- and deca- vanadate anions may also contribute to the stabilization of the enzyme conformation optimal for crystallization of the Ca²⁺-transport ATPase.

Tight binding of oligovanadates with inhibition of enzymic activity was also observed in the case of several phosphotransferase enzymes, such as phosphofructokinase (Choate & Mansour, 1979), adenylate kinase (DeMaster & Mitchell, 1973; Pai *et al.*, 1977); hexokinase (Climent *et al.*, 1981) and phosphorylase (Soman *et al.*, 1983), and may represent a fairly general property of phosphohydrolases and phosphotransferases.

Neither the structure of the bound polyvanadates nor the location and properties of their binding site(s) on the Ca²⁺-transport ATPase are established. Equilibrium binding studies revealed a single site per Ca²⁺-transport ATPase for monovanadate (Medda & Hasselbach, 1983; P. Csermely, S. Varga & A. Martonosi, unpublished work) that is presumably identical with the binding site for orthophosphate (site 1). Binding of

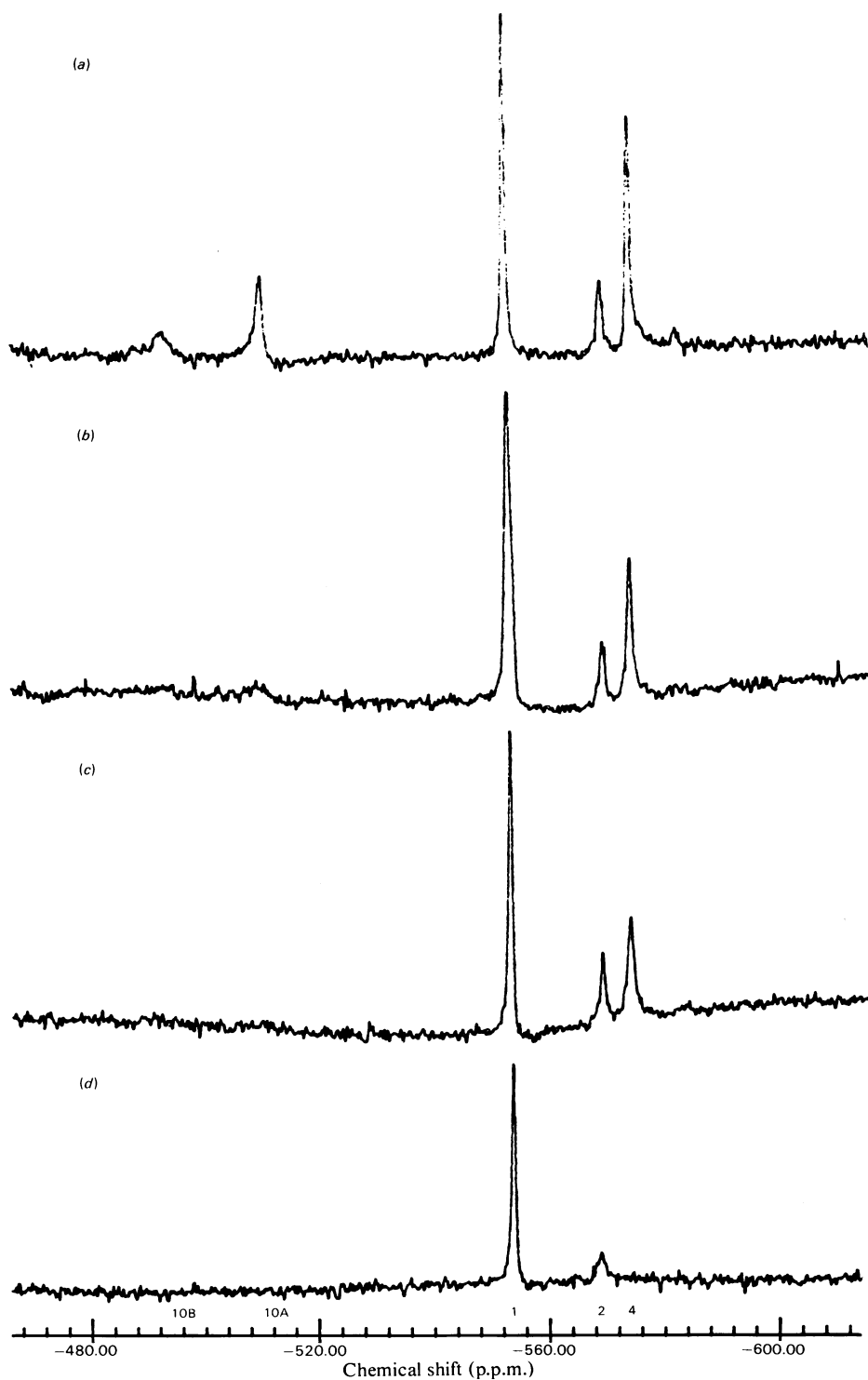


Fig. 5. *Effect of sarcoplasmic-reticulum vesicles on the spectra of vanadate solutions*

To solutions of 0.1 M-KCl/5 mM-MgCl₂/0.5 mM-EGTA/10 mM-imidazole, pH 7.4, containing a mixture of monovanadate and decavanadate at final concentrations of 1 mM each, sarcoplasmic-reticulum vesicles were added to final concentrations of 3.5 mg (b), 5.0 (c) and 20 mg (d) of protein/ml. Sample (a) did not contain sarcoplasmic reticulum. The spectra were measured within 10 min after mixing, at 25°C.

vanadate at this site inhibits ATPase activity and Ca^{2+} transport (Pick, 1982; Pick & Karlsh, 1982), and blocks the cleavage of the Ca^{2+} -transport ATPase at the T_2 site (Dux & Martonosi, 1983b). Because of its high affinity, this site is presumably saturated under the conditions used in the present studies, but, owing to the relatively low sensitivity of n.m.r., would not be detected. Covalent labelling of the Ca^{2+} -transport ATPase with fluorescein isothiocyanate, which inhibits ATP binding (Pick & Karlsh, 1980; Pick & Bassilian, 1981), did not interfere with the binding of monovanadate to the high-affinity site.

It is likely that oligovanadates are also able to occupy the monovanadate-binding site, because decavanadate reproduces all effects of monovanadate at low vanadate concentrations. In addition, oligovanadates are bound at a second set of site(s) on the Ca^{2+} -transport ATPase (site 2); binding of decavanadate at this site interferes with covalent labelling of the Ca^{2+} -transport ATPase by fluorescein suggesting that the binding site for decavanadate overlaps with the binding site for ATP (P. Csermely, S. Varga & A. Martonosi, unpublished work).

The greater affinity of the highly charged oligovanadates as compared with monovanadate to the enzyme is best explained if electrostatic forces play an important role in the interaction; this in turn suggests that the binding site for oligovanadates in the Ca^{2+} -transport ATPase, as in adenylate kinase (Pai *et al.*, 1977), may be rich in basic amino acids. Although mixtures of lysine, arginine and histidine (1 mM each) did not influence the n.m.r. spectrum of oligovanadates, guanidine precipitates decavanadate from solutions (Soman *et al.*, 1983), with selective disappearance of decavanadate lines from the n.m.r. spectrum.

Binding of oligovanadates to the sarcoplasmic reticulum is accompanied by the broadening and disappearance of the corresponding n.m.r. signals. The line broadening of the bound decavanadate is extreme, and even the averaging of half a million transients did not yield a recognizable signal of the decavanadate bound to sarcoplasmic reticulum. Therefore, solution n.m.r. studies are not likely to yield information about the structure of membrane-bound vanadate polyanions, and such information must be sought by solid-phase n.m.r. or by X-ray crystallography. Several factors may contribute to the broadening of the n.m.r. signal: the restricted mobility of the bound vanadium, distortion of the symmetry around the vanadate nucleus due to binding, exchange between different forms of vanadate, the physical heterogeneity of the medium etc. (Rehder, 1982). The observed broadening is not attributable to field heterogeneity, since it was not produced by glycogen, Ludox

particles and liposomes at density comparable with that of sarcoplasmic reticulum.

Exchange between bound and free decavanadate is likely to contribute to the disappearance of decavanadate signal in the presence of sarcoplasmic-reticulum vesicles, since with excess vanadate some decavanadate signal re-appeared in the supernatant after ultracentrifugal sedimentation of the microsomal particles. The possibility of line broadening by exchange makes difficult the quantitative evaluation of decavanadate binding to microsomal particles from n.m.r. spectra.

The observations outlined in the present paper emphasize the complex composition of vanadate solutions generally used for biochemical studies, and stress the need for consideration of the binding of di-, tetra- and decavanadate species at multiple and overlapping sites in the interpretation of the effects of vanadate on the Ca^{2+} -transport ATPase of sarcoplasmic reticulum.

This work was supported by Research Grants AM 26545 and RR 01317 from the National Institutes of Health, Grant PCM 84-03679 from the National Science Foundation and a grant-in-aid from the Muscular Dystrophy Association. The n.m.r. measurements and spectral analysis were performed at the N.I.H. Resource for Multi Nuclei NMR and Data Processing, Department of Chemistry, Bowne Hall, Syracuse University, Syracuse, NY, U.S.A. We express our thanks to Dr. M. Bruch and Mr. Wang de Hua for assistance with the n.m.r. measurements.

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