The Partial Purification of Sodium-Plus-Potassium Ion-Dependent Adenosine Triphosphatase from the Gills of *Anguilla anguilla* and its Inhibition by Orthovanadate

By MICHAEL V. BELL and JOHN R. SARGENT

N.E.R.C. Institute of Marine Biochemistry, Aberdeen AB1 3RA, Scotland U.K.

(Received 27 October 1978)

1. ($\text{Na}^+ + \text{K}^+$)-dependent ATPase was partially purified from eel gills by a procedure in which the microsomal fraction of crude preparations of chloride cells was selectively extracted with sodium dodecyl sulphate. 2. The microsomal specific activity was increased 2-fold during optimal treatment with detergent. 3. The final preparation (56% pure) had a specific activity of $341 \mu\text{mol}$ of ATP hydrolysed/h per mg of protein and a turnover number of 3560 min^{-1} . The number of ouabain-binding sites equalled the number of sites phosphorylated by ATP. 4. Both sodium orthovanadate and ouabain inhibited the purified preparation more than the microsomal fraction, vanadate being more effective on an equimolar basis than ouabain. 5. Inhibition by orthovanadate was not enhanced at 28 mM-as compared with 1 mM- MgCl_2 and was not reversed by β -adrenergic agonists (cf. Josephson & Cantley (1977) *Biochemistry* 16, 4572–4578). 6. Of various other metallic oxyanions tested only niobate proved an effective inhibitor of the enzyme although this anion was less effective than orthovanadate. 7. Orthovanadate partially inhibited phosphorylation of the enzyme by ATP in the presence of 28 mM- MgCl_2 .

($\text{Na}^+ + \text{K}^+$)-dependent ATPases from numerous tissues, including mammalian skeletal muscle, kidney and heart, the rectal gland of *Squalus* and the electric organ of *Electrophorus* are strikingly inhibited by sodium orthovanadate (Josephson & Cantley, 1977; Cantley *et al.*, 1977; Quist & Hokin, 1978). Cantley *et al.* (1977) suggested that a possible biological role for vanadium, an essential dietary factor in mammals at least (Hopkins & Mohr, 1974), may be to regulate ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity and, therefore, the 'sodium pump' activity of cells. It has, in fact, long been known that the clinical effects of digitalis and vanadate are similar (Gamgee & Larmuth, 1877). Vanadate was thoroughly established as a powerful peripheral vasoconstrictor in numerous species by Jackson (1911–1912), a finding recently confirmed for a marine teleost by Bell *et al.* (1979).

The above findings have obvious implications for marine organisms. Vanadium is a natural constituent of sea water generally considered to be present largely as the orthovanadate anion at a reported concentration of approx. 50 nM (Brewer, 1975). At least one group of marine organisms, the ascidians, actively concentrates vanadium, together with the other group-V(A) elements, niobium and tantalum (Goodbody, 1974). Vanadate is transported across the branchial network of ascidians by a ouabain-sensitive process (Rummel *et al.*, 1966). The present

report, part of a study of the mechanism of uptake and biological effects of vanadium in marine organisms, describes some effects of metallic oxyanions on a preparation of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase partially purified from the gills of a marine fish, *Anguilla anguilla*.

Materials and Methods

Radioisotopes

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ triethylammonium salt (18–20 Ci/mmol) and $[\text{G-}^3\text{H}]\text{ouabain}$ (14.7 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Chemicals

The disodium salt of ATP (grade 1, by phosphorylation of adenosine, vanadate-free), ouabain, noradrenaline, adrenaline, isoproterenol, 2-mercaptoethanol, imidazole and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. Acrylamide, bis-(NN' -methylenebisacrylamide), Temed ($\text{NNN}'\text{N}'$ -tetramethylethylenediamine), Coomassie Brilliant Blue R250 and SDS were purchased from Bio-Rad Laboratories, Bromley, Kent, U.K. Basic Fuchsin was purchased from Searle, High Wycombe, Bucks., U.K.

Abbreviation used: SDS, sodium dodecyl sulphate.

Sodium orthovanadate, reagent grade, was purchased from BDH Chemicals, Poole, Dorset, U.K., as were niobium pentachloride and tantalum pentachloride. The pentachlorides were converted into sodium niobate and sodium tantalate as follows: 10g of the pentachloride was added slowly with stirring to a 5-fold molar excess of NaOH solution and left overnight to allow complete reaction. The resulting suspension was heated to dissolve the precipitate and cooled to re-form the precipitate, which was then centrifuged, washed with cold water and re-crystallized twice from hot water. The final solids were dried *in vacuo* over P_2O_5 and small samples were then dried at 200°C to constant weight to determine the water of crystallization.

All other chemicals were AnalaR grade from BDH. All solutions were prepared in glass-distilled water. Stock solutions of sodium orthovanadate, sodium niobate and sodium tantalate were freshly prepared each day at concentrations of 10mM; the pH values of the stock solutions were not adjusted.

Animals

Sexually mature eels (*Anguilla anguilla*) were netted in a freshwater loch (Loch of Skene) near Aberdeen, immediately transferred to a seawater aquarium at 8–12°C and kept there for at least 1 month before being killed.

Preparation of $(Na^+ + K^+)$ -dependent ATPase

Fish were killed by decapitation and the gills immediately perfused *in situ* with a chilled solution containing 150mM-NaCl/2mM- K_2SO_4 /0.11mM- $(NH_4)_2SO_4$ /25mM-Hepes/12mM-NaOH/2mM- Na_2EDTA /5.5mM-glucose, pH7.6. The gill arches were excised, blotted dry and the gill epithelium scraped off the underlying cartilage with a scalpel. All subsequent operations were carried out at 0–2°C. The gill scrapings (4–5g wet wt.) were suspended in 25ml of perfusion medium, stirred magnetically for 5min and filtered through Nylon gauze of rectangular mesh 106 μ m square (Henry Simon, Stockport, U.K.). The residue was suspended in 25ml of fresh medium, subjected to two strokes in a Dounce homogenizer, stirred for 5min as before and again filtered through gauze. The combined filtrates were centrifuged at 6750 g_{av} -min and the resulting supernatant discarded to leave a pellet of crude chloride cells. At least 90% of the original $(Na^+ + K^+)$ -dependent ATPase in the scrapings was present at this stage.

The crude pellet was suspended in 30ml of 0.32M-sucrose containing 1mM- Na_2EDTA , adjusted to pH8.0 with 0.1M-NaOH, and homogenized with a glass-in-Teflon homogenizer (0.125–0.175mm clearance) operated at speed 6 (500–11000 rev./min

range) with a Tri-R model K43 drive: 12 complete strokes were used. The homogenate was diluted to 300ml with the above buffer and fractionated as follows. The suspension was centrifuged at 6500 g_{av} -min to give a pellet of cell debris and a supernatant. The latter was centrifuged at 300000 g_{av} -min to give a biphasic pellet consisting of firmly packed mitochondria (tan) and loosely packed microsomes (white). The loose microsomal material was swirled off in a small vol. of the supernatant and homogenized in about 30ml of this fraction (six strokes, conditions as above). The rehomogenized microsomal fraction was mixed with the remaining supernatant and centrifuged at $2.4 \times 10^6 g_{av}$ -min to give a microsomal pellet. This fraction contained about 85% of the enzyme activity present in the original homogenate.

All pellets from the subcellular fractionation were suspended in a solution containing 5mM-imidazole / 1mM- Na_2EDTA / 1mM-2-mercaptoethanol / 0.32M-sucrose, brought to pH7.0 with 1M-HCl, at a concentration of 1.5–3.0mg of protein/ml (three complete strokes, speed 3, conditions as above). The microsomal fraction was stored at –20°C.

Detergent treatment of microsomes

The microsomal fraction was slowly thawed at room temperature and diluted to give a protein concentration of 1.5mg/ml with 5mM-imidazole/1mM- Na_2EDTA / 1mM-2-mercaptoethanol / 0.32M-sucrose, pH7.0. Each preparation was then titrated with detergent to determine the optimal protein:detergent ratio. Then 0.5ml samples of diluted microsomal fraction were taken in 2ml MSE centrifuge tubes and treated with sodium dodecyl sulphate, prepared in the above buffer, at concentrations between 0.02 and 0.07% (w/v). The suspension was briefly vortex-mixed immediately after adding the detergent then left on ice for 30min with periodic shaking before centrifuging at $2.4 \times 10^6 g_{av}$ -min. The resulting pellets were suspended in 0.5ml of buffer and vortex-mixed. All pellet and supernatant fractions were assayed for enzyme activity and protein and the optimal detergent concentration then selected for treatment of the remaining microsomes. This was invariably close to 0.05% (w/v) of sodium dodecyl sulphate, which is near the critical micellar concentration of the detergent under these conditions.

The treatment of bulk microsomal fraction was exactly as above using 8–10ml portions in boiling tubes to mimic the geometry of the 2ml centrifuge tubes as closely as possible. After detergent treatment and centrifugation the supernatant and some loose pellet material were decanted off. The firm pellet material was resuspended in a small volume of the original buffer (no detergent) with homogenization (six strokes, speed 3, conditions as above) to give a

protein concentration of at least 1.5mg/ml. This fraction was stored at -20°C and retained at least 90% of its activity after 1 month.

Enzyme assays

The enzyme was assayed at 30°C by the automated method of Dale *et al.* (1975) under conditions where the reaction rate was independent of time and proportional to enzyme concentration. The basic assay contained 1 mM-MgCl₂/1 mM-Na₂ATP/100 mM-NaCl/30 mM-imidazole/HCl, pH 7.0 at 30°C, and between 8 and 16 µg of microsomal protein/ml or 1.5–3 µg of the purified enzyme preparation/ml. Enzyme activity was calculated from the mean of duplicate assays carried out in the presence and absence of 20 mM-KCl. Enzyme activity in the absence of K⁺ was taken to be due to one or more Mg²⁺-dependent ATPases. Under these assay conditions this activity was less than 20% of the total microsomal ATPase activity and less than 5% of the final ATPase activity present. Units of (Na⁺+K⁺)-dependent ATPase are expressed as µmol of phosphate released/h.

Phosphorylation with [γ -³²P]ATP

The method used was that of Hokin *et al.* (1973) as detailed by Sargent & Thomson (1974), except that 100 µg of bovine serum albumin was included in each assay to ensure complete precipitation of all the protein by the trichloroacetic acid.

Na₂ATP (2 µCi; 25 µM) was used in each assay and the difference taken between assays carried out in the presence of 100 mM-NaCl and 20 mM-KCl.

Binding of [³H]ouabain

A modification of the method of Hansen (1971) as described by Sargent & Thomson (1974) was used: 1 µCi of [G-³H]ouabain (14.7 Ci/mmol) was used per 1.0 ml assay. All values were corrected for non-specific binding by subtracting the value obtained when the assays were carried out in the presence of 20 mM-KCl substituted for 100 mM-NaCl.

Polyacrylamide-gel electrophoresis

Electrophoresis was carried out in tubes 13 cm long and 5 mm internal diameter with a 10% gel (as described by Weber & Osborn, 1969). The sample (30–40 µg of protein) was applied after solubilization in an equal volume of 2% (w/v) SDS/2% (w/v) 2-mercaptoethanol/0.01 M-sodium phosphate, pH 7.0, at 100°C for 10 min. Electrophoresis was performed for 5½ h, starting with 1 mA/gel for the first hour and 8 mA/gel thereafter. The gels were then stained for protein in 0.04% (w/v)

Coomassie Blue R250/10% (v/v) acetic acid/25% (v/v) propan-2-ol for 16 h, and were destained in 10% (v/v) acetic acid/5% (v/v) propan-2-ol. Glycoprotein components were detected by staining with the method of Zacharius *et al.* (1969).

Densitometry

A Vitatron TLD densitometer (Vitatron, Dieren, The Netherlands) was used with a slit width of 0.25 mm, a span setting of 1000, a scan speed of 0.5 cm/min and a chart speed of 2 cm/min. For gels stained with Coomassie Blue a red filter transmitting light of more than 600 nm wavelength was used. No filter was used for gels stained for glycoprotein. A Hewlett-Packard model 3380A integrator was used for quantifying the resultant peaks.

Protein determinations

Protein was measured by the method of Lowry *et al.* (1951) in the presence of 0.5% (w/v) sodium deoxycholate, with bovine serum albumin as a standard.

Results

The (Na⁺+K⁺)-dependent ATPase from the gills of seawater-adapted eels (*Anguilla anguilla*) has already been characterized (Sargent & Thomson, 1974; Bell *et al.*, 1977), but has yet to be isolated in a pure state. The purification procedure used here, essentially that of Jørgensen (1974), is simple and reproducible. It involves preparation of tissue fractions enriched in 'chloride cells', isolation of a microsomal fraction from these cells and extraction of the latter with detergent. The final preparation had a specific activity of 341 ± 22 µmol/h per mg of protein (mean ± s.e., *n* = 4) (Table 1), the highest so far obtained from gill tissue. The estimated purity of the final preparation, based on quantitative densitometry of SDS/polyacrylamide gels stained with Coomassie Blue, was 56 ± 5% (mean ± s.e., *n* = 4).

The yield of the enzyme, 70 ± 8% (mean ± s.e., *n* = 4), includes a 2-fold activation factor [2.02 ± 0.18 (mean ± s.e., *n* = 9)] arising from detergent treatment of the microsomal fraction. The specific activity of the enzyme (Table 1) and its specific phosphorylation with ATP (Table 5) were both purified some 6-fold from the microsomal fraction to the final preparation. We infer that phosphorylation of the enzyme with ATP, like the enzyme activity, is activated 2-fold when microsomes are treated optimally with detergent. This result is consistent with the microsomal fraction containing a random population of 'inside-out' and 'inside-in' membrane vesicles with only the former having enzyme sites accessible to ATP. Treatment with

detergent presumably renders all enzyme sites accessible to ATP. Sodium deoxycholate and Lubrol could also be used to activate the microsomal enzyme activity and plots of total enzyme activity against detergent concentration yielded maxima close to the critical micellar concentration of the detergent. However, sodium dodecyl sulphate gave the greatest purification so this detergent was used in the routine purification of the enzyme.

A further feature of the purification scheme was that treatment of microsomes with detergent in the presence of sucrose-enhanced protein solubilization and thus improved the purity of the final preparation. Such an effect of sucrose on protein solubilization has been noted previously by Thompson & Bachelard (1970) using Triton X-100, but the mechanism remains obscure (Helenius & Simons, 1975).

When examined by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels the final preparation contained two major components: a protein of approx. mol. wt. 90000 and a glycoprotein of approx. mol. wt. 45000 (cf. Sargent & Thomson, 1974). The turnover number of the final preparation, based on its specific activity and specific phosphorylation with ATP (Tables 1 and 5), was $3560 \pm 390 \text{ min}^{-1}$ (mean $\pm$ S.E., $n = 4$).

Table 2 shows the effects of both sodium orthovanadate and ouabain on the microsomal and the final preparations. Orthovanadate inhibits the

purified preparation substantially more than the microsomal fraction, e.g. $0.1 \mu\text{M}$ -vanadate inhibits these activities by 55 and 24% respectively. It has been reported that orthovanadate inhibition of $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity is greater at higher MgCl_2 concentrations, optimal inhibition occurring at 28 mM-MgCl_2 (Josephson & Cantley, 1977). This effect was not observed here. Although 28 mM-MgCl_2 by itself inhibited the enzyme by approx. 50%, the actual inhibition due to orthovanadate at this concentration of MgCl_2 was not increased over that observed at 1 mM-MgCl_2 .

Inhibition by ouabain, like that by orthovanadate, is more effective on the purified than on the microsomal preparation although the steroid glycoside is less effective than orthovanadate on an equimolar basis (Table 2). The microsomal enzyme activity is rather resistant to ouabain in the sense that complete inhibition is only observed at 1 mM -ouabain. Binding studies with $[^3\text{H}]\text{ouabain}$ (66 nM) carried out in the presence and absence of 20 mM-KCl (see the Materials and Methods section) showed that the microsomes bound $49.7 \pm 5.3 \text{ pmol}$ of ouabain/mg of protein (mean $\pm$ S.E., $n = 4$) and the final preparation bound $973 \pm 44 \text{ pmol}$ of ouabain/mg of protein (mean $\pm$ S.E., $n = 2$). Thus the ratio of potassium-sensitive ouabain-binding sites to sites phosphorylated with ATP (Table 5) was approximately 1:1 with the purified enzyme (cf. Sargent & Thomson, 1974). The same

Table 1. Partial purification of $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase from the gills of *Anguilla anguilla*

Full details are given in the Materials and Methods section. Results are means $\pm$ S.E. of four separate preparations starting with 1.0 g wet wt. of 'scrapings' from the gill epithelium. During treatment of microsomes with SDS the specific activity increased 2.02 ± 0.18 -fold (mean $\pm$ S.E., $n = 9$). —, Not determined.

	Total units	Specific activity (units/mg of protein)	Yield (%)	Purity (%)
Homogenate	490 ± 65 (4)	10.6 ± 1.0 (4)	100	—
Microsomes	405 ± 59 (4)	54.0 ± 2.0 (4)	82 ± 3 (4)	—
Pellet from treatment with SDS	331 ± 24 (4)	341 ± 22 (4)	70 ± 8 (4)	56 ± 5 (4)

Table 2. Inhibitory effects of sodium orthovanadate and ouabain on $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase from the gills of *Anguilla anguilla*

The microsomal fraction and the detergent-purified preparation were assayed as described in the Materials and Methods section using $8\text{--}16 \mu\text{g}$ of microsomal protein/ml or $1.5\text{--}3 \mu\text{g}$ of the SDS pellet preparation/ml per assay in the presence of the concentrations of sodium orthovanadate and ouabain shown. The specific activities of the various preparations are shown in parentheses. Data are expressed as percentage inhibition.

Orthovanadate (M)	Inhibition (%)		Ouabain (M)	Inhibition (%)	
	Microsomes (49)	SDS pellet (274)		Microsomes (62)	SDS pellet (274)
10^{-3}	100	100	10^{-3}	100	100
10^{-4}	95	100	10^{-4}	78	100
10^{-5}	66	88	10^{-5}	40	91
10^{-6}	39	70	10^{-6}	11	62
10^{-7}	24	55	10^{-7}	0	6
10^{-8}	0	12	10^{-8}	0	0

ratio for the microsomal enzyme activity assayed under the same conditions was 1:7 suggesting an apparent deficiency or unavailability of ouabain-binding sites on microsomes. Increasing the concentration of [³H]ouabain to 0.1mM dramatically increased the quantity of ouabain bound to microsomes by approx. 1000-fold. However, this binding was now non-specific in the sense that there was no significant difference between ouabain bound in the presence and absence of 20mM-KCl. Thus, at a concentration of ouabain (1mM) sufficient to inhibit the microsomal enzyme activity completely, the glycoside appears to be bound extensively and non-specifically to sites on microsomes other than (Na⁺+K⁺)-dependent ATPase.

It has been reported that β -adrenergic compounds antagonise the inhibition of (Na⁺+K⁺)-dependent ATPase by orthovanadate (Hudgins & Bond, 1977; Josephson & Cantley, 1977). Table 3 shows that adrenaline, noradrenaline and isoproterenol by themselves have slight stimulatory and inhibitory effects on the microsomal and purified preparations respectively. Only slight reversal of vanadate inhibition in the purified preparation occurred with the compounds tested, even at concentrations 100 times that of vanadate.

Table 4 summarizes the effects of selected metallic oxyanions on (Na⁺+K⁺)-dependent ATPase activi-

ties in the microsomal and purified preparations. Niobium and tantalum were chosen as group-V(A) elements. Molybdate, tungstate and arsenate were tested because these anions, like orthovanadate, are tetrahedral. Inhibitory effects were noted for all the anions tested although, niobate apart, the effects were slight. On an equimolar basis niobate is much less effective than orthovanadate although both anions inhibit the purified enzyme more than the microsomal enzyme. Some technical difficulties were encountered in testing the various anions. Tantalate precipitated at concentrations of 0.1mM and above. At 1mM tungstate interfered with colour development in the P_i assay and, as expected, arsenate produced strong colour development at 1mM. Despite these difficulties it is certain that none of the anions tested, niobate included, approached the effectiveness of orthovanadate, which appears to be a unique inhibitor of (Na⁺+K⁺)-dependent ATPase.

In an effort to define the mechanism of enzyme inhibition by orthovanadate we tested the effects of the anion on the phosphorylation of the enzyme by ATP. Table 5 shows that phosphorylation of the microsomal fraction is not influenced significantly by 10 μ M-orthovanadate in the presence of either 1mM- or 28mM-MgCl₂. Slight inhibitory effects were noted with the purified preparation at 1mM-MgCl₂ and very significant inhibition occurred with the purified

Table 3. *Effects of adrenergic compounds on (Na⁺+K⁺)-dependent ATPase activity and its inhibition by orthovanadate* Assays were carried out as detailed in the Materials and Methods section with 8–16 μ g of microsomal protein/ml or 1.5–3 μ g of the SDS pellet preparation/ml per assay. Values in parentheses after microsomal and detergent-purified preparations indicate specific activities. Data are expressed as percentages of control activity.

	Activity (% of control)			
	Microsomes (62)		SDS pellet (274)	
	Control	+10 μ M-Vanadate	Control	+10 μ M-Vanadate
No additions	100	52	100	30
0.1 mM-Noradrenaline	104	63	87	37
0.1 mM-Adrenaline	107	57	84	40
0.1 mM-Isoproterenol	106	49	95	35

Table 4. *Effects of various metallic oxyanions on the activity of (Na⁺+K⁺)-dependent ATPase from the gills of *Anguilla anguilla** Assays were carried out as detailed in the Materials and Methods section with 8–16 μ g of microsomal protein/ml or 1.5–3 μ g of the SDS pellet preparation/ml per assay. Values in parentheses indicate the specific activities of the preparations. Data are expressed as percentage inhibition. —, Not determined.

Concn. (M)	Inhibition (%)					
	Microsomes (62)			SDS pellet (274)		
	NbO ₄ ³⁻	TaO ₄ ³⁻	MoO ₄ ²⁻	WO ₄ ²⁻	AsO ₄ ³⁻	
10 ⁻³	53	—	21	—	—	73
10 ⁻⁴	25	—	0	27	10	42
10 ⁻⁵	15	4	0	0	0	19
						7
						15
						0

preparation at 28mM-MgCl₂. This inhibition was dependent on the specific activity of the preparation tested and did not exceed 50%.

Discussion

Two different approaches have been used in recent years to purify (Na⁺+K⁺)-dependent ATPase from a number of sources. Hokin and his co-workers have used the non-ionic detergent Lubrol to solubilize the enzyme and subsequently remove contaminating proteins by density gradient centrifugation and (NH₄)₂SO₄ fractionation, obtaining a preparation from *Squalus* rectal gland of specific activity 1500 (Hokin *et al.*, 1973) and a preparation from *Electrophorus* electric organ of specific activity 1900 (Perrone *et al.*, 1975). Jørgensen (1974) used the alternative procedure of solubilizing contaminating microsomal proteins with SDS leaving the (Na⁺+K⁺)-dependent ATPase in particulate membrane fragments which were further purified by density gradient centrifugation. The enzyme isolated from dog kidney by this procedure had a specific activity of 2000 (Jørgensen, 1974).

The final preparation obtained here was only some 50% pure and efforts to improve the purity were unsuccessful. In particular, efforts to solubilize the enzyme from microsomes with Lubrol under controlled conditions induced marked and variable instability of enzyme activity. The final preparation had a specific activity approaching 400 and a purity of 50% indicating that the pure (Na⁺+K⁺)-dependent ATPase from fish gills will have a specific activity approaching 800. This is much lower than the values of 1500–2000 for other (Na⁺+K⁺)-dependent ATPases with the exception of the value of 800 for the enzyme recently isolated from brine shrimps by Peterson *et al.* (1978). Thus the turnover numbers of (Na⁺+K⁺)-dependent ATPases vary from about 3000–3500 min⁻¹ for the brine shrimp enzyme (Peterson *et al.*, 1978) and the enzyme studied here, to about 6000–9000 min⁻¹ for the enzymes from mammalian kidney (Jørgensen, 1974), *Squalus* rectal gland (Hokin *et al.*, 1973) and *Electrophorus* electric organ (Perrone *et al.*, 1975).

The [³H]ouabain binding to microsomes found in the present study was proportionally much lower than expected from the specific enzymic activity and the extent of phosphorylation of the preparation with ATP. Most reported values for [³H]ouabain binding, including that in a previous study of the gill enzyme (Sargent & Thomson, 1974), have been determined on preparations treated with detergent. It is possible, therefore, that detergent treatment renders the ouabain-binding sites more accessible to the ligand or alters the membrane in other ways including removing phospholipid. This effect may underlie the different sensitivities of microsomal and

detergent-treated enzymes to ouabain (Table 2). The sensitivity of the (Na⁺+K⁺)-dependent ATPase from mouse ascites cells is known to be modified by other membrane constituents (Lelievre *et al.*, 1976) and Charnock *et al.* (1977) have reported that phospholipase A treatment altered the ouabain-binding characteristics of a membrane preparation from bovine brain. The presence of other ligands affected ouabain binding to normal and lipid-depleted (Na⁺+K⁺)-dependent ATPase from rabbit kidney in different ways (Goodman & Wheeler, 1978). These findings point to an involvement of the phospholipid matrix of biomembranes in the interaction of ouabain and (Na⁺+K⁺)-dependent ATPase activity. We note that, at the concentration of ouabain (0.1 mM) required here to inhibit the microsomal enzyme almost completely, there was a very large molar excess of ouabain bound to the preparation.

In considering the effects of orthovanadate on (Na⁺+K⁺)-dependent ATPase the results here confirm the original work of Josephson & Cantley (1977) and Cantley *et al.* (1977) that this anion is a very powerful inhibitor of the enzyme. However, inhibition of the enzyme from eel gills differs somewhat from that of the enzymes from mammalian kidney and mammalian heart (Cantley *et al.*, 1977), *Squalus* rectal gland and *Electrophorus* electric organ (Quist & Hokin, 1978). The gill enzyme is less sensitive to vanadate inhibition than enzymes from these sources and inhibition is not enhanced by high concentrations of MgCl₂. Moreover, inhibition of the gill enzyme is not reversed by β -adrenergic agonists as has been reported for the other enzymes. It has been suggested that β -adrenergic agonists interact chemically with orthovanadate to relieve inhibition of (Na⁺+K⁺)-dependent ATPase (Josephson & Cantley, 1977; Cantley *et al.*, 1978a; Quist & Hokin, 1978), a situation that clearly does not occur at the concentrations tested here (Table 3). It is noteworthy, however, that the powerful vasoconstricting effects of 1 μ M-orthovanadate on perfused eel gills are completely prevented by prior administration of 0.1 mM-isoproterenol (Bell *et al.*, 1979). Such a result could, of course, be accounted for simply by the β -adrenergic agonist altering haemodynamics in the perfused organ, e.g. opening blood by-passes, as well as by direct interaction with orthovanadate at its immediate site of action, presumably (Na⁺+K⁺)-dependent ATPase. Should this direct interaction occur *in vivo* then it is certain that it cannot be reproduced *in vitro* with the (Na⁺+K⁺)-dependent ATPase preparation used here.

Any mechanism for vanadate inhibition of (Na⁺+K⁺)-dependent ATPase must account for the specificity of the anion in inhibiting or otherwise related ATPases. Orthovanadate has no inhibitory effects on mitochondrial F₁-ATPase or actomyosin

Table 5. *Effects of orthovanadate on the phosphorylation of (Na⁺+K⁺)-dependent ATPase with [γ -³²P]ATP*

Assays were carried out on the different preparations shown as detailed in the Materials and Methods section. Between 50 and 100 μ g of microsomal protein and 20–40 μ g of the SDS pellet material were used per assay. Data are expressed as pmol of [γ -³²P]ATP reacting/mg of protein $\pm$ S.E. (number of determinations in parentheses). —, Not determined.

	Phosphorylation (pmol of [γ - ³² P]ATP reacting/mg of protein)			
	1 mM-MgCl ₂		28 mM-MgCl ₂	
	Control	+10 μ M-Vanadate	Control	+10 μ M-Vanadate
Microsomes 1	274 $\pm$ 4 (3)	279 $\pm$ 5 (3)	—	—
SDS pellet 1	1420 $\pm$ 75 (3)	1191 $\pm$ 35 (3)	—	—
Microsomes 2	315 $\pm$ 50 (2)	297 $\pm$ 98 (2)	351 $\pm$ 53 (2)	313 $\pm$ 84 (2)
SDS pellet 2	1694 $\pm$ 138 (2)	1492 $\pm$ 104 (2)	1673 $\pm$ 201 (2)	841 $\pm$ 118 (2)

ATPase (Josephson & Cantley, 1977) or on the Mg²⁺-dependent, Ca²⁺-dependent and (Ca²⁺+Mg²⁺)-dependent ATPases from sarcolemma (Quist & Hokin, 1978). However, orthovanadate inhibits dynein ATPases from several sources (Nagata & Flavin, 1978).

Orthovanadate has been considered an analogue of orthophosphate; both anions are tervalent and tetrahedral and the V–O bond length (0.156 nm) is close to the P–O bond length (0.165 nm) (Clark, 1973). Molybdate and tungstate are tetrahedral and bivalent and do not inhibit the enzyme (Table 4). However, arsenate is both tetrahedral and tervalent and the As–O bond length (0.178 nm) is not far removed from the P–O bond length, but the anion does not inhibit the enzyme, at least at 0.1 mM (Table 4). It is difficult, therefore, to account for the marked inhibitory effects of orthovanadate strictly in terms of this anion being an analogue of orthophosphate, especially since the latter anion itself does not inhibit the enzyme significantly (Robinson *et al.*, 1978). We note, however, a difference between orthophosphate and orthovanadate which is that the latter can exist as a quadrivalent tetrahedral trimer, HV₃O₁₀^{4–}, at physiological pH values (Clark, 1973). The tetrahedral anion niobate forms even longer polymers of uncertain chain length ($n = 6$ –18) at pH values in the region of 9 and the tendency to polymerize is even more pronounced with tantalate (Fairbrother, 1967). This accounts for the relative insolubility of tantalate and, presumably, its inability to inhibit the enzyme. In short the ability to inhibit (Na⁺+K⁺)-dependent ATPase can be correlated with the ability of tetrahedral anions to form short polymers at physiological pH values which, in the unique case of orthovanadate, are linear quadrivalent trimers. We suggest that Na₂MgHV₃O₁₀^{4–} is potentially a unique analogue of the actual substrate of (Na⁺+K⁺)-dependent ATPase, Na₂MgATP^{4–}. Some support for this idea is found in Table 5 where 10 μ M-orthovanadate partially inhibited phosphorylation of the enzyme in the presence of 28 mM-MgCl₂. Although phos-

phorylation was not significantly inhibited at 1 mM-MgCl₂ (Table 5), when the overall enzyme reaction is markedly inhibited (Table 2), it may be unwise to equate conditions in the non-kinetic phosphorylation assay with those in the kinetic assay for enzyme activity. It remains to be seen whether kinetic studies can unravel the potentially complex interactions between the different anionic forms of orthovanadate, Na⁺, K⁺, Mg²⁺, ATP, ADP and P_i and (Na⁺+K⁺)-dependent ATPase.

Note Added in Proof (Received 29 January 1979)

Since this paper was accepted for publication a detailed analysis of the interactions of orthovanadate with (Na⁺+K⁺)-dependent ATPase has been published (Cantley *et al.*, 1978b).

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