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COMMENTARY

VANADATE AND ITS SIGNIFICANCE IN BIOCHEMISTRY AND PHARMACOLOGY

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In 1977 Cantley and co-workers [1] successfully purified from 'Sigma Grade' ATP a potent inhibitor of $(Na^+ + K^+)$-ATPase which was found to be identical with sodium orthovanadate (Na_3VO_4). Vanadate was confirmed to be present in commercially available ATP prepared from muscle but not in ATP isolated from yeast [2, 3]. As it was found in many mammalian tissues and inhibited several $(Na^+ + K^+)$-ATPase preparations in as low as micromolar or even nanomolar concentrations, many investigators soon saw a role for vanadate as an endogenous regulator of the sodium pump.

Cardiac glycosides are known to inhibit specifically $(Na^+ + K^+)$-ATPase activity, the biochemical equivalent of the 'sodium pump', in minute concentrations after binding reversibly but with high affinity to this enzyme. Their main pharmacological effect, the time-dependent increase in the force of contraction of the heart, is thought to be the consequence of this specific inhibition of the sodium pump [4], although this latter concept is not generally accepted (for review see [5]). When it was discovered (again) that vanadate increased the force of contraction of an isolated papillary muscle without stimulation of the β-adrenoceptor [6], it was even thought to be a possible endogenous cardiac glycoside. Although we soon learned that vanadate certainly is not an endogenous cardiac glycoside, this hypothesis and subsequent findings stimulated some of the most exciting basic research on the physiological, biochemical and pharmacological role of vanadate.

In the following we would like to concentrate mainly on the cardiac effects and significance of vanadate; for a general review the reader is referred to several recently published, excellent articles [7–9].

Complex chemical properties

Vanadium, a group V element (mol. wt. 50.9), belongs to the first transition series and forms compounds mainly in valence states +3, +4 and +5. It readily changes its oxidation state. In the presence of O_2, air or in oxygenated blood (i.e. under conditions prevalent in experiments using physiological methods) or oxidizing agents (e.g. diamide), vanadium is always in the +5 oxidation state irrespective of the nature of the original compound. In the presence of reducing agents such as ascorbic acid, vanadium compounds are in the +4 oxidation state. Furthermore, vanadium can undergo changes in charge, depending on the ambient pH. Under physiological conditions at pH 7.4, vanadium in the +5 oxidation is in the form of an anion, vanadate (VO_3^-; VO_4^{3-} or its isopolyanions), while vanadium in the +4 oxidation state at this pH is predominantly in the cationic form, vanadyl (VO^{2+}). V^{5+}-Cationic and V^{4+}-anionic forms only exist under strongly basic or acid conditions, respectively, and need not be considered in this discussion [10–12]. Vanadate tends to aggregate, even at low concentrations of 1 mM, to form a trimer or a tetramer. At lower pH, orange-coloured decavanadate predominantes and acts as a powerful oxidant of aldehydes, catechols, olefins and sulfhydryls. Vanadium chemistry on the whole is similar to that of phosphorus [9].

Biological significance of vanadium

The trace element vanadium is essential for rats and chicks [13]. In these animals vanadium deprivation may cause reduced growth, increased plasma cholesterol levels, impaired reproductive performance and severe disorganization of the cells of the epiphysis with subsequent bone malformation. However, the dietary intake has to be below 10–100 ng vanadium/g food in rats or chicks in order to cause deficiency symptoms. At present, it is not known whether vanadium is an essential element for man as well. The normal food intake in hospital diets was measured recently as *ca.* 20–50 ng/g and the mean intake 12.4–28 μg/day [14]. This would cause deficiency symptoms in some animals. Apparently humans need even less vanadium.

Persons occupationally exposed to vanadium eliminate high amounts in the urine. The cystine content found in their fingernails is significantly reduced [15] and when intoxicated (possible with high doses only), gastrointestinal symptoms and a green tongue appeared [16].

Recently it has been reported that mean plasma concentrations in manic-depressive illness (0.296 ± 0.036 μM) were increased as compared to controls (0.192 ± 0.025 μM) [17, 18]. Furthermore, in

uraemia, serum vanadium concentrations were measured as 1.23 ± 0.16 μg/ml (~25 μM) and in control subjects as 0.4 ± 0.04 μg/ml [19]. If confirmed, these findings would have great clinical implications. Vanadate is known to be an extremely potent vasoconstricting agent (also of the coronary arteries), and patients with progressive renal insufficiency usually suffer from hypertension and coronary heart disease. There is a serious problem of reliably measuring serum vanadium concentrations, however. Serum vanadium levels in man have been reported as 400 ng/ml when determined by atomic absorption spectrometry [19] or emission spectography [20]. These methods are, however, not sufficiently sensitive and probably render mere artefacts [21].

It is accepted today that the true values in control persons are about or below 0.1–1 ng/ml (~2–20 nM) when neutron activation analysis is used for determination. Therefore, the results of the above-mentioned clinical studies have to be taken with the greatest caution, and present knowledge of vanadium toxicity in man is very scarce indeed. Apparently workers chronically exposed to vanadium with mean serum concentrations of 2.9 ng/ml (range 2.5–52.4 ng/ml) did not show overt signs of toxicity [15]. Vanadium contents of animal or human organs are much higher than serum levels. In unexposed human liver, for instance, 2.5–13.4 ng V/g wet tissue have been determined. This will be discussed later.

Thus the biological significance of the trace element vanadium in man is not well understood at present. Serum and tissue concentrations in rats [22] with and without vanadium feeding are well known. There are hardly any toxic effects unless gross overdosage occurs.

Pharmacological effects of vanadate

Vanadate (NH_4VO_3, Na_3VO_4) in micromolar concentrations has a potent dose-dependent positive inotropic effect on ventricular cardiac preparations in cat [6], guinea pig [23], rat [24], rabbit [25] and dog [26]. In fact, this vanadate-stimulated increase in the force of contraction has been reported as early as 1912 by Jackson [26] and has been described in the following way: ". . . vanadium acted very much in the same manner as digitalis . . .". As mentioned, the positive inotropic effect of vanadate is a direct effect and is not due to β-adrenoceptor stimulation or inhibition of phosphodiesterase [27]. It is stable for the time of exposure and can be washed out easily [6]. In atrial preparations of guinea pig, cat and beef [23] but not in rat [28], vanadate produced a negative inotropic effect. $(Na^+ + K^+)$-ATPase preparations isolated from atrial and ventricular muscle were inhibited in the same manner and by identical concentration ranges ($IC_{50} \sim 0.6$ μM Na_3VO_4). Thus the different effects of vanadate on the force of contraction in atrial and ventricular muscles are certainly not due to different effects on $(Na^+ + K^+)$-ATPase activity as measured in isolated enzyme preparations [23].

Vanadate in micromolar concentrations is a potent diuretic agent in the rat [29, 30]. In cats and dogs, however [31, 32], it causes a pronounced reduction of urine production accompanied by a rise in blood pressure due to marked vasoconstriction. This vasoconstrictor effect, probably due to its action on vascular smooth muscle [33, 35], prohibits its use as an inotropic agent in intact animals. Vanadate causes coronary vasoconstriction and a subsequent fall in cardiac output under these circumstances (Fig. 1). Thus the mode of action of vanadate at the cellular level, i.e. as a possible endogenous regulating agent, is easier to evaluate and also more interesting.

Biochemical effects of vanadate

In isolated cardiac cell membranes of those animal species tested, vanadate inhibits the $(Na^+ + K^+)$-ATPase activity in the micromolar range. This is true for human cardiac cell membranes, too. In rat cardiac $(Na^+ + K^+)$-ATPase preparations, vanadate is even more potent than ouabain. The $(Na^+ + K^+)$-ATPase activity is supposed to represent the biochemical equivalent of the 'sodium pump' [36]. Thus one would expect to find an inhibition of active Na^+ and K^+ transmembranous transport caused by vanadate in intact cardiac preparations. In fact, in human erythrocytes, vanadate inhibits the $(Na^+ + K^+)$-ATPase and $^{86}Rb^+$-uptake (used as a measure of $^{42}K^+$-uptake) [37]. In these cells, dinitrostilbene disulphonate (DITS) inhibits the uptake of ^{48}V-vanadate. This suggests that vanadate is taken up into the red cells by the anion-exchange system and that vanadate inhibits the $(Na^+ + K^+)$-ATPase from the cytoplasmic side [37]. In intact cardiac cells from the rat, however, the active uptake of $^{86}Rb^+$ or $^{42}K^+$ is not inhibited by vanadate [28]. In contrast, vanadate stimulated $(^{86}Rb^+ + K^+)$-uptake in cultured myocardial cells of the rat [38]. Vanadate-induced alterations of beating frequency and of speed of contraction in these cells are paralleled by a stimulation of $(^{86}Rb^+ + K^+)$-uptake of up to 75%. Maximal stimulation is obtained at concentrations of 0.1–1 mM vanadate. As this stimulation can be inhibited by ouabain [38], it is probably due to an increased activity of $(Na^+ + K^+)$-ATPase. Thus in purified cardiac cell membranes, vanadate inhibits $(Na^+ + K^+)$-ATPase, whereas in intact cardiac preparations stimulation is observed. This makes it very unlikely that the positive inotropic effect of vanadate is connected with $(Na^+ + K^+)$-ATPase, and it furthermore warns us not to accept too readily experimental results from subcellular particles as representative of the intact cell.

Recently, Werdan *et al.* [39] discovered an ouabain-like inhibition of potassium uptake (up to 50%) combined with a decrease of cellular potassium (up to 20%) by vanadate (10^{-4}–10^{-3} M) in heart non-muscle cells obtained from neonatal guinea pigs and chick embryos. In heart muscle cells and non-muscle cells prepared from neonatal rats, as well as in Girardi human heart cells, however, a vanadate-induced stimulation of potassium uptake (up to 100%) combined with a rise in cellular potassium (up to 20%) without significant alteration of cellular sodium could be confirmed [39]. A slight increase of $^{22}Na^+$-influx was measured in cultured beating rat heart muscle cells and in Girardi human heart cells at these vanadate concentrations. In cultured rat heart muscle cells, vanadate is taken up in an energy-independent manner by a saturable transport system ($K_m \sim 60$ μM, $V \sim 200$ pmole/mg protein per min at 37°). Analysis of intracellular binding of ^{48}V-van-

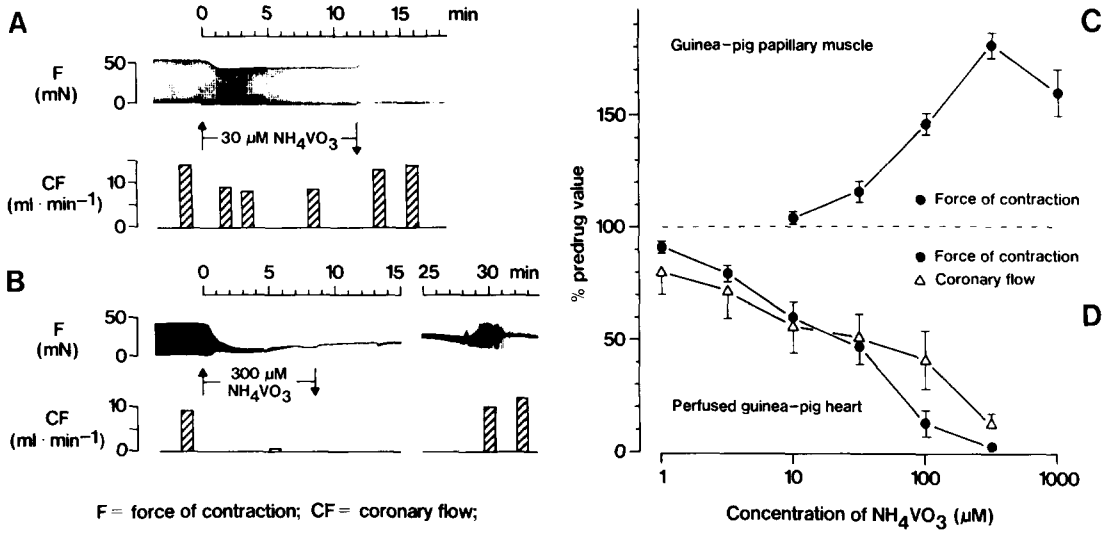

Fig. 1. Effects of ammonium vanadate (NH_4VO_3) on the force of contraction and coronary flow of perfused whole hearts (A, B, D) and on the force of contraction of papillary muscles (C) isolated from guinea pigs. All preparations were electrically driven at frequencies of 4 Hz (A, B, D) or 1 Hz (C). A: Experiment illustrating the effect of 30 μM NH_4VO_3 on the force of contraction (F) and coronary flow (CF). NH_4VO_3 was present between ↑ and ↓. B: Experiment similar to that shown in A but with 300 μM NH_4VO_3. An episode of ventricular fibrillation occurred between the 28th and 31st min of the experiment. C: Concentration–response curve illustrating the positive inotropic effect of NH_4VO_3 in papillary muscles. The pre-drug value (100%) was 1.14 ± 0.13 mN, $n = 9$. D: Concentration–response curves illustrating the NH_4VO_3-induced decrease in the force of contraction and in the coronary flow in perfused hearts. The pre-drug values were 100.4 ± 10.7 mN (●) and 12.8 ± 2 ml/min (△), $n = 4$. The concentration–response curves in C and D were obtained cumulatively. The time of exposure to each concentration of NH_4VO_3 was 10 min. Values are means ± S.E.M. [34].

adate revealed a curvilinear Scatchard plot indicating more than one binding site. Maximal binding was found to amount to *ca.* 3×10^9 molecules of vanadate per cell [38].

Intracellular vanadium is probably bound to cytoplasmic proteins as vanadyl (+4) [40–42], which does not inhibit cardiac $(Na^+ + K^+)$-ATPase [12]. Although ^{48}V-vanadate is bound to isolated cell membranes and inhibits $(Na^+ + K^+)$-ATPase activity in these broken cell membrane preparations, it does not inhibit the sodium pump in intact cells, because it is reduced to vanadyl intracellularly. It has been found that isolated cell membranes (cardiac and liver, etc.) can reduce vanadate to vanadyl [43]. Probably a specific enzyme, a NADH-vanadate reductase [44], which belongs to the plasma membrane oxidoreductase complex [45, 46] is responsible for this change in valency. In the human red cell, glutathione reduces vanadate [47]. Glutathione is present in most cells in high concentrations (3–5 mM); therefore endogenous vanadium probably exists mostly as vanadyl, at least in some cells. This reduction certainly explains the 'resistance' of $(Na^+ + K^+)$-ATPase to vanadate in intact myocardial muscle cells.

In vivo, $(Na^+ + K^+)$-ATPase is not inhibited by vanadate—at least not in contracting rat heart ventricular strips or in rat heart cells. Nevertheless, vanadate has been used as an interesting tool by those who work with the enzyme. Apparently isolated $(Na^+ + K^+)$-ATPase contains rather specific binding sites for vanadate ($K_1 = 4$ nM, $K_2 = 0.5$ μM) which may be identical to the binding sites for ATP [48, 49]. Recently attempts to form crystalline arrays of pure membrane-bound renal $(Na^+ + K^+)$-ATPase in the presence of vanadate have been successful [50]. A discussion of these very exciting procedures is, however, beyond the scope of this article.

Adenylate cyclase and vanadate

In isolated rat fat cell membranes [51] as well as in guinea-pig cardiac membranes [52], a concentration-dependent stimulation of adenylate cyclase by vanadate has been measured. Again vanadyl is rather ineffective [12]. Further work has clearly demonstrated a vanadate-caused rise in intracellular cAMP concomitant with the positive inotropic effect in intact cat papillary muscle [27] and without inhibition of phosphodiesterase activity. The nature of this stimulation has been clarified recently [53]. The effect of vanadate on adenylate cyclase is mediated through the nucleotide regulatory protein of this enzyme complex, and vanadate may act by a mechanism similar to that of fluoride, although the exact mechanism of both anions (fluoride and vanadate) on the nucleotide regulatory protein is slightly different [53].

The positive inotropic effect of vanadate might be the result of the increased intracellular cAMP. However, in contrast to isoprenaline, the cAMP-elevating action of vanadate seemed to be less pronounced than the positive inotropic effect. Furthermore, vanadate did not abbreviate the isometric contraction, which is a characteristic of β-adrenoceptor agonists (for instance isoprenaline). Rather, vanadate prolonged the isometric contrac-

tion [27]. Adenylate cyclase activity of both guinea pig atrial and ventricular muscles is stimulated by vanadate [25]. Therefore it is rather unlikely that the positive and/or negative inotropic effects are connected to the elevation of cAMP. Thus at present there is no proven causal relationship between the effects of vanadate on $(Na^+ + K^+)$-ATPase, adenylate cyclase and the observed positive inotropy.

Vanadate, Ca^{2+}-ATPase and other ATPases

At a free Ca^{2+} concentration of 1.3×10^{-6} M, the Ca^{2+}-ATPase isolated from pig heart sarcoplasmic reticulum is half-maximally inhibited by 10^{-5} M vanadate [54]. At a similar concentration, vanadate produces its positive inotropic effects in isolated cardiac preparations. Under those conditions, 10^{-8}–10^{-7} M vanadate, however, stimulates SR Ca^{2+}-ATPase from pig heart. The consequences of these findings in respect to the augmented force of concentration are not understood.

Ca^{2+}-ATPases from plasmalemma, sarcoplasmic reticulum and mitochondria from rabbit heart are inhibited by vanadate too [55]. Cardiac myofibrillar ATPase is also inhibited by vanadate. These actions and the inhibition of Ca^{2+} transport of the sarcoplasmic reticulum [55] would suggest that intracellular vanadate produces negative rather than positive inotropic effects. Thus again one must assume that vanadate does not enter the cardiac cell as vanadate. The intracellular reducing milieu may cause the change to vanadyl, as described earlier.

In dialysed squid axons, vanadate inhibits with high affinity ($K_{1/2} \sim 7\ \mu$M) the ATP-dependent uncoupled Ca^{2+} efflux but does not affect the Na_0-dependent Ca^{2+} efflux in the absence of ATP [56]. Since externally applied vanadate was without this effect, it has been concluded that the sites with which vanadate interacts are internally located. Internal potassium increases the affinity of these sites for vanadate [56]. These findings agree with the conditions in human red cells, where vanadate, present internally, inhibits active Ca^{2+}-efflux [57].

A number of ATPases are inhibited by vanadate: myosin-ATPase [58], dynein-ATPase, *Saccharomyces cerevisiae* plasma membrane Mg^+-ATPase, plasma membrane ATPase from *Neurospora crassa* and several others (for review see [9]). For all of these enzymes, however, vanadate has to enter the cell as vanadate, which has not been demonstrated. Furthermore, the inhibition of the different ATPases does not explain the positive inotropic effects seen *in vitro* and in perfused rabbit hearts [55].

The force of contraction increases if either the sensitivity of the contractile proteins for calcium or the intracellular availability for calcium is augmented. It has been demonstrated that vanadate has no effect on the Ca^{2+} sensitivity of tension or stiffness [59]. There is evidence, however, that vanadate does affect a superficially located calcium pool which is also influenced by verapamil [24]. The vanadate effects on the force of contraction can be abolished by Mn^{2+}. Vanadate failed to affect the force of the first contraction observed after a brief quiescent period in both rat and guinea pig cardiac preparations, indicating that it does not influence the calcium pool related to 'post-rest potentiation' of contraction. The negative inotropic effect of vanadate observed in Ca^{2+}-restored guinea pig preparations was associated with an inhibition of the characteristic slow action potentials [24]. From these and other experiments, it was concluded that vanadate increases the intracellular availability of Ca^{2+} by affecting a superficial calcium pool. The exact mechanism of action is unknown, however.

Insulin-mimetic effects of vanadium compounds

Insulin modifies the movements of Na^+ and K^+ ions across the cell membrane, with the consequence of increased cellular K^+ and decreased cellular Na^+ concentrations. These effects have been attributed to an insulin-induced enhancement of active Na^+, K^+-transport, to a hyperpolarization of the cell membrane, as well as to alterations of the membrane permeabilities for Na^+ and K^+ ions [60–62]. For beating rat heart muscle cells and cardiac non-muscle cells from guinea pigs, stimulation of active K^+-influx by insulin—as measured by $^{86}Rb^+$—occurs. In rat heart muscle cells, this insulin action is mimicked by vanadate: vanadate stimulates $(^{86}Rb^+ + K^+)$-uptake in a concentration-dependent manner, increases cellular K^+ and decreases cellular Na^+ contents. The stimulation is abolished by ouabain. In the presence of saturating concentrations of insulin, vanadate produces no further stimulation of $(^{86}Rb^+ + K^+)$-uptake. In addition, the ouabain-independent $^{22}Na^+$-influx is increased to some extent by vanadate [39]. These effects can be attributed to an increased activity of the sodium pump and an increased permeability of the cell membrane for Na^+ ions.

The stimulatory action of vanadate on $(^{86}Rb^+ + K^+)$-uptake has been also observed in rat ventricular strips, in cardiac non-muscle cells from the rat, and in Girardi human heart cells [39]. Also the vanadate-induced increase in cellular K^+ and decrease in cellular Na^+ in rat skelatal muscle [63], and the hyperpolarization of the cell membrane of mouse diaphragm in the presence of vanadate [64] are compatible with an insulin-like activation of the sodium pump by vanadate.

The insulin-mimetic effect of vanadium compounds is not only restricted to active Na^+/K^+-transport: glucose transport and glucose oxidation are also stimulated by vanadate in an insulin-like manner in rat adipocytes [65, 66], rat skeletal muscle. [63], rat heart muscle cells and non-muscle cells, and in Girardi human heart cells [39]. With respect to glucose oxidation, vanadium in the +4 valence state represents the active form [66]. Given the lack of definitive knowledge concerning the molecular mechanism of insulin action, consideration of the possible mechanisms underlying the insulin-mimetic effects of vanadium compounds is necessarily speculative, and the biological significance non-established [9, 65, 66].

Paradoxically, vanadium compounds either inhibit or stimulate active Na^+/K^+-transport, depending on the cell type and tissue tested [39]. A possible explanation for this apparent discrepancy may lie in the fact that vanadium in the +5 valance state represents an effective inhibitor of $(Na^+ + K^+)$-ATPase [40], while vanadium in the +4 valence state mimics the action of insulin [66]. Taking into account the

enzymatic and/or non-enzymatic reduction of vanadium(V) to vanadium(IV) within the cell, the following hypothesis might explain the action of vanadium compounds on active Na^+/K^+-transport: depending on the different cellular capacities for the reduction of vanadate(V) to vanadyl(IV) in different cell types, vanadium may either act more as an ouabain-like inhibitor, or more as an insulin-mimetic stimulator of active Na^+/K^+-transport. If vanadate can act as an inhibitor of $(Na^+ + K^+)$-ATPase in the intact cell, the insulin-mimetic action is blocked because it depends on non-suppressed enzymatic activity of $(Na^+ + K^+)$-ATPase. If vanadate is unable to act as an inhibitor of $(Na^+ + K^+)$-ATPase, due to the reduction to vanadyl(IV), the insulinmimetic effect clearly appears [39]. The experimental data are consistent with this hypothesis: if vanadate stimulates ($^{86}Rb^+ + K^+$)-uptake—as shown for rat heart muscle cells—it also enhances, like insulin, the uptake of the glucose analogue 2-deoxy-D-glucose. If vanadate inhibits ($^{86}Rb^+ + K^+$)-uptake—as shown for cardiac non-muscle cells from guinea pigs—it also fails to stimulate glucose uptake.

Conclusions

After its discovery as an inhibitor of $(Na^+ + K^+)$-ATPase, vanadate was claimed to be a physiological inhibitor of the sodium pump, representing some sort of endogenous cardiac glycoside. It was also the hope that vanadate could help to clarify the yet unproven mechanism of positive inotropic action of cardiac glycosides in the heart. Both suggestions have failed due to several reasons.

While cardiac glycosides are specific inhibitors of $(Na^+ + K^+)$-ATPase, vanadate can be characterized as an unspecific modifier of ATP-consuming enzymes. In addition, it also affects other enzymes not involved in ATP-splitting. As only vanadium in the +5 valence state inhibits $(Na^+ + K^+)$-ATPase, and as living cells are able to reduce vanadium(V) to a variable extent, the effect of vanadate on active Na^+/K^+-transport in the living cell is not simply predictable from experiments with membrane preparations: Na^+/K^--transport may be unaffected, inhibited or even enhanced by vanadate, the latter being due to an insulin-like stimulation of the sodium pump by vanadium in the +4 valance state. A general role of vanadate as an endogenous cardiac glycoside can therefore be ruled out.

The mechanism of positive inotropic action of vanadate in heart preparations still remains an open question. However, a glycoside-like action, in general, can be excluded. As vanadate also exerts insulin-like effects, the positive inotropic action of insulin should be kept in mind. Independent of the underlying mechanism, however, the positive inotropic action of vanadate can only be demonstrated *in vitro*; *in vivo*, the pronounced vanadate-induced constriction of the coronary arteries dominates, always producing a negative 'inotropic' effect in the intact animal.

Thus vanadate does not play a role as a positive inotropic agent in the intact animal; the mechanism of its positive inotropic action in cardiac preparations is unclear; its supposed role as an endogenous regulator of the sodium pump seems questionable. In analogy to the still-unanswered question why vanadium is essential, we must state that the physiological role of vanadate in heart function and metabolism is, at present, far from being understood.

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