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Molybdate and tungstate act like vanadate on glucose metabolism in isolated hepatocytes

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In rat hepatocytes, molybdate and tungstate inactivate glycogen synthase by a mechanism independent of Ca^{2+} and activate glycogen phosphorylase by a Ca^{2+}-dependent mechanism. On the other hand, both molybdate and tungstate increase fructose 2,6-bisphosphate levels and counteract the decrease in this metabolite induced by glucagon. These effectors do not directly modify 6-phosphofructo-2-kinase activity, even though they partially counteract the inactivation of this enzyme induced by glucagon. These effects are related to an increase on the glycolytic flux, as indicated by the increase in L-lactate and CO_2 production and the decrease in glucose 6-phosphate levels in the presence of glucose. All these effects are similar to those previously reported for vanadate, although molybdate and tungstate are less effective than vanadate. These results could indicate that molybdate, tungstate and vanadate act on glucose metabolism in isolated hepatocytes by a similar mechanism of action.

INTRODUCTION

In recent years, many reports have been focused on the study of the insulin-like effects of vanadate on glucose metabolism. Thus it has been reported that this compound activates glucose transport and oxidation in several isolated tissues [1–3], and it has been described that vanadate raises fructose 2,6-bisphosphate [Fru(2,6)P_2] levels and activates glycolysis in hepatocytes isolated from normal [4] or diabetic [5] rats. In addition, several other reports indicate that oral administration of vanadate to diabetic rats normalizes hepatic glucose metabolism and glucose blood levels and restores insulin-responsiveness of target tissues [6–12]. However, we have also demonstrated that vanadate provokes an inactivation of glycogen synthase and a concomitant activation of glycogen phosphorylase in isolated hepatocytes from both normal [13] or diabetic [14] rats. Then, at least in isolated hepatocytes, vanadate seems to provoke a wide range of responses on glucose metabolism.

Tungstate and molybdate have some similar characteristics to vanadate. Vanadium(V), Mo(VI) and W(VI) have the same electronic configuration (d^0). They all form iso- and hetero-polyanions, which present octahedral co-ordination spheres [15]. It has also been reported that these compounds exert some biological effects similar to those provoked by vanadate. In this way, it has been reported that vanadate, molybdate and tungstate increased the rate of quantum jumps in *Limulus* ventral photoreceptors in the dark. Likewise, these compounds activated phosphodiesterase from retinal rod outer segments of *Limulus* in the dark [16], although, whereas vanadate and molybdate are able to activate membrane adenylate cyclase from some tissues [17–19], tungstate does not activate this enzyme [18].

In the present paper we have studied the effects of molybdate and tungstate on some key regulatory points of hepatocyte glucose metabolism. Moreover, we have compared the results obtained with those observed for vanadate, in order to relate the effects of these three compounds. The results indicate that vanadate, molybdate and tungstate act in a similar manner on glucose metabolism in isolated hepatocytes, suggesting that these three compounds act through a similar mechanism of action.

MATERIALS AND METHODS

Incubation of the cells

Suspensions of isolated hepatocytes from 24 h-starved rats (180–250 g) were prepared as indicated in [20]. Cells were resuspended in Krebs–Ringer bicarbonate buffer (pH 7.4) free of glucose or any gluconeogenic precursor unless otherwise stated. When stated, Ca^{2+} was omitted from this medium and 1 mM-EGTA was added 20 min before any further treatment of the cells. Samples [3–5 ml, (4–5) $\times 10^6$ cells/ml] were incubated at 37 °C with continuous shaking. When stated, cells were pre-incubated for 30 min with 16 mM-lactate/4 mM-pyruvate, or for 20 min with 10 mM-glucose before treatment with the effectors.

To determine glycogen synthase and phosphorylase activities, at the end of incubations hepatocytes were centrifuged and pellets were immediately homogenized with a Polytron homogenizer (setting 6–7) in 300 μl of ice-cold 10 mM-Tris/HCl buffer (pH 7.4) containing 150 mM-KF, 15 mM-EDTA, 0.6 M-sucrose, 1 mM phenylmethanesulphonyl fluoride, 1 mM-benzamidine, 25 μg of leupeptin/ml and 50 mM-β-mercaptoethanol. Finally, cell homogenates were centrifuged at 10000 ***g*** for 20 min at 4 °C, and the supernatants were used to determine glycogen synthase and phosphorylase activities and total protein.

Enzyme and metabolite assays

Glycogen synthase activity ratio was measured by the low-glucose 6-phosphate/high-glucose 6-phosphate method as described in [21]. Glycogen phosphorylase *a* activity was measured as in [22]. Protein was determined by the biuret method, modified as in [23].

To determine Fru(2,6)P_2 levels and 6-phosphofructo-2-kinase (PFK-2) activity, 0.1 ml samples of cell suspension were frozen in liquid N_2. To measure Fru(2,6)P_2, frozen samples were thawed in 0.6–0.8 ml of 54 mM-NaOH and were then heated at 80 °C for 5 min. Fru(2,6)P_2 levels were determined as described by Van Schaftingen *et al.* [24]. To determine PFK-2 activity, frozen samples were thawed in 0.1 ml of buffer containing 20 mM-potassium phosphate (pH 7.0), 10 mM-EDTA and 100 mM-KCl, and the activity was determined as described in [25].

Abbreviations used: Fru(2,6)P_2, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase.

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To determine glucose 6-phosphate and ATP levels, neutralized $HClO_4$ extracts of cell pellets were used. Glucose 6-phosphate was determined enzymically as described in [26], and ATP was measured by h.p.l.c. as in [27]. L-Lactate levels were determined enzymically in 12000 *g* cell supernatants as in [28]. To determine $^{14}CO_2$ production, hepatocytes incubated in the presence of 10 mM-[U-^{14}C]glucose were treated as described in [4].

Materials

Sodium orthovanadate, sodium tungstate, disodium molybdate, glucagon and [U-^{14}C]glucose were from Sigma. All other reagents were analytical grade.

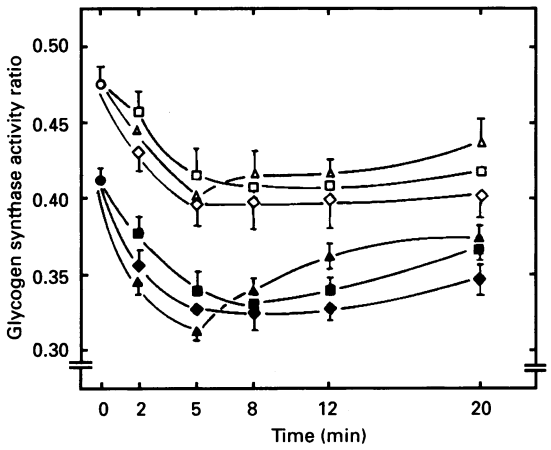

Fig. 1. Time-dependent effects of molybdate, tungstate and vanadate on glycogen synthase activity ratio

Hepatocytes were resuspended in a Krebs–Ringer medium with Ca^{2+} (◆, ■, ▲) or in a medium without Ca^{2+} and supplemented with 1 mM-EGTA (◇, □, △). Then cells were incubated for the indicated times with 2 mM-vanadate (◆, ◇), 5 mM-tungstate (■, □) or 10 mM-molybdate (▲, △). Results are means ± S.E.M. for five separate experiments.

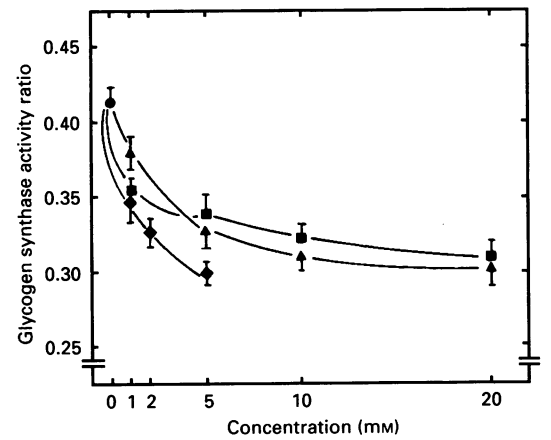

Fig. 2. Dose-dependent effects of molybdate, tungstate and vanadate on glycogen synthase activity ratio

Hepatocytes resuspended in a Krebs–Ringer medium with Ca^{2+} were treated for 5 min with increasing concentrations of molybdate (▲), tungstate (■) or vanadate (◆). Results are means ± S.E.M. for four separate experiments.

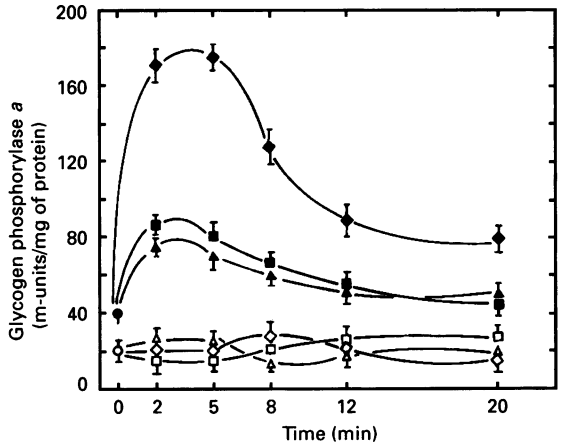

Fig. 3. Time-dependent effects of molybdate, tungstate and vanadate on glycogen phosphorylase *a* activity

Hepatocytes were resuspended in a Krebs–Ringer medium with Ca^{2+} (▲, ■, ◆) or in medium without Ca^{2+} and supplemented with 1 mM-EGTA (△, □, ◇). Then cells were incubated with 10 mM-molybdate (▲, △), 5 mM-tungstate (■, □) or 2 mM-vanadate (◆, ◇) for the indicated times and glycogen phosphorylase *a* activity was measured. Results are means ± S.E.M. for five independent experiments.

RESULTS

Effects of molybdate and tungstate on glycogen synthase activity

Incubation of rat hepatocytes with vanadate causes the inactivation of glycogen synthase [13,14]. When these cells were incubated in the presence of 10 mM-molybdate or 5 mM-tungstate, a time-dependent decrease in glycogen synthase activity ratio was also observed (Fig. 1). The effect was maximal after 5–8 min of incubation. Specific activity measured at high (10 mM) glucose 6-phosphate concentration remained constant throughout the incubation period. At these concentrations neither molybdate nor tungstate significantly modified intracellular ATP levels after 10 min of incubation (control, 2.4 ± 0.2 μmol/g; molybdate, 2.0 ± 0.2 μmol/g; tungstate, 2.3 ± 0.1 μmol/g; means ± S.E.M. for 3 experiments). Incubation with 2 mM-vanadate provoked a similar inactivation of glycogen synthase (Fig. 1). The effects of molybdate, tungstate and vanadate on glycogen synthase activity ratio were also concentration-dependent (Fig. 2). Half-maximal effects of these effectors were obtained at concentrations between 1 and 5 mM.

Effects of vanadate on glycogen synthase are independent of the presence of Ca^{2+} ([13]; Fig. 1). Therefore we next investigated whether molybdate and tungstate were also able to modify glycogen synthase activity ratio in Ca^{2+}-depleted cells. As shown in Fig. 1, both 10 mM-molybdate and 5 mM-tungstate decreased glycogen synthase activity ratio in these conditions. These results indicate that all these effectors act on glycogen synthase activity by a mechanism which does not require Ca^{2+}.

Effects of molybdate and tungstate on glycogen phosphorylase activity

Vanadate activates glycogen phosphorylase [13,14]. Similarly, when hepatocytes were incubated in the presence of 10 mM-molybdate or 5 mM-tungstate, a time-dependent increase in glycogen phosphorylase *a* activity was observed (Fig. 3). This effect was maximal for both effectors after 2 min of incubation, and basal levels were practically reached after 20 min of incubation. Vanadate was more potent than molybdate or tungstate (Fig. 3). In Ca^{2+}-depleted cells none of these effectors were able

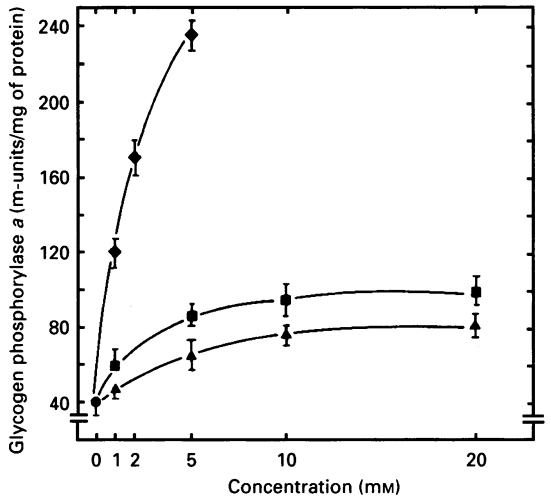

Fig. 4. Dose-dependent effects of molybdate, tungstate and vanadate on glycogen phosphorylase activity

Hepatocytes were incubated for 2 min with the indicated concentrations of molybdate (▲), tungstate (■) and vanadate (◆). Results are means ± S.E.M. for four different experiments.

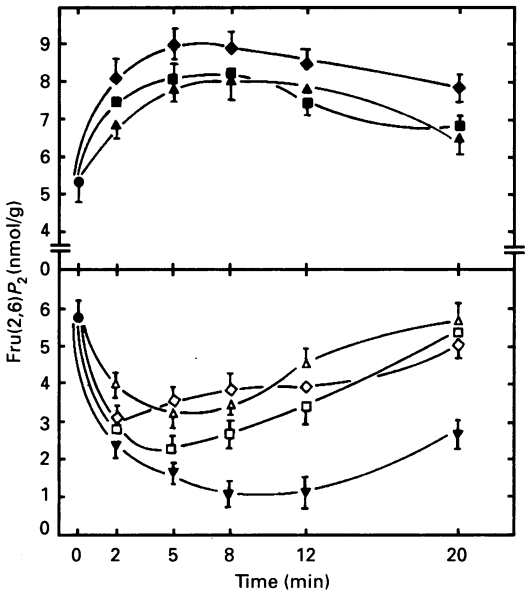

Fig. 5. Effects of molybdate, tungstate and vanadate on Fru(2,6)P_2 levels: counteraction of the action of glucagon

Hepatocytes were resuspended in a Krebs–Ringer medium with Ca^{2+} and were then preincubated for 30 min with 16 mM-lactate/4 mM-pyruvate before addition of the effectors. (*a*) Cells were incubated, for the indicated times, with 2 mM-vanadate (◆), 5 mM-tungstate (■) or 10 mM-molybdate (▲). Results are means ± S.E.M. for five different experiments. (*b*) Hepatocytes were incubated, for the indicated times, with 10 nM-glucagon (▼), or with 10 nM-glucagon plus 2 mM-vanadate (◇), 10 nM-glucagon plus 5 mM-tungstate (□), or 10 nM-glucagon plus 10 mM-molybdate (△). Results are means ± S.E.M. for four independent experiments.

to activate glycogen phosphorylase (Fig. 3), indicating that they act on glycogen phosphorylase activity through a Ca^{2+}-dependent mechanism.

Glycogen phosphorylase activation induced by molybdate, tungstate and vanadate was also dose-dependent (Fig. 4). Half-maximal effects were reached at concentrations of molybdate or

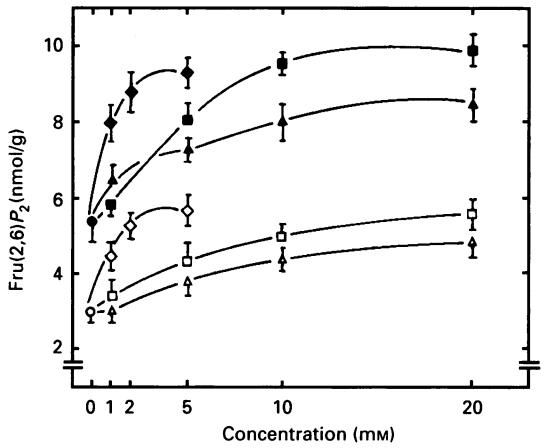

Fig. 6. Dose-dependent effects of molybdate, tungstate and vanadate on Fru(2,6)P_2 levels

Hepatocytes were resuspended in a Krebs–Ringer medium with Ca^{2+} (▲, ■, ◆) or in a medium without Ca^{2+} and supplemented with 1 mM-EGTA (△, □, ◇). Then cells were incubated for 8 min with the indicated concentrations of molybdate (▲, △), tungstate (■, □) or vanadate (◆, ◇). Results are means ± S.E.M. for three different experiments.

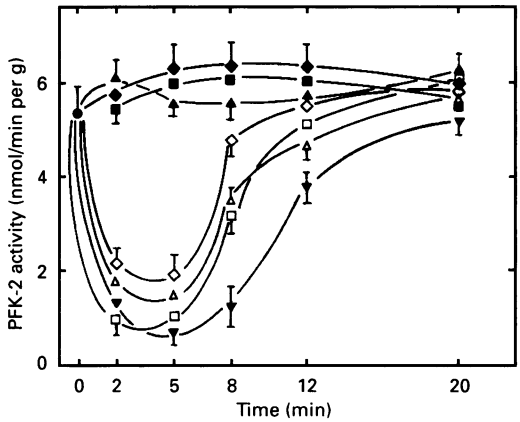

Fig. 7. Counteraction of glucagon effects on PFK-2 activity by molybdate, tungstate and vanadate

Hepatocytes were preincubated in the presence of 16 mM-lactate/4 mM-pyruvate for 30 min. Then they were incubated for the indicated times with 10 mM-molybdate (▲), 5 mM-tungstate (■) or 2 mM-vanadate (◆). Cells were also treated with 10 nM-glucagon (▼) or with 10 nM-glucagon plus 10 mM-molybdate (△), 10 mM-glucagon plus 5 mM-tungstate (□) or 10 nM-glucagon plus 2 mM-vanadate (◇). Results are mans ± S.E.M. for four separate experiments.

tungstate between 1 and 5 mM. Vanadate was more potent than tungstate, and tungstate more than molybdate.

Effects of molybdate and tungstate on Fru(2,6)P_2 levels

Since vanadate is able to increase intracellular Fru(2,6)P_2 levels [4,5], we next studied whether molybdate and tungstate were able to produce the same effect. As shown in Fig. 5(*a*), 5 mM-tungstate, 10 mM-molybdate and 2 mM-vanadate caused a great increase on Fru(2,6)P_2 levels in hepatocytes incubated in the presence of 16 mM-lactate/4 mM-pyruvate. The increase was in all cases time-dependent. Maximal effects were reached after 5 min of incubation with the effectors. The increase was also concentration-dependent (Fig. 6). Tungstate and molybdate produced half-maximal effects at concentrations between 1 and

Table 1. Effects of molybdate, tungstate and vanadate on L-lactate and $^{14}CO_2$ production and glucose 6-phosphate concentrations

Hepatocytes were resuspended in a Krebs–Ringer medium. To determine $^{14}CO_2$ production, cells were incubated for 60 min with 10 mM-[U-^{14}C]glucose in the absence or in the presence of 10 mM-molybdate, 5 mM-tungstate or 2 mM-vanadate, and $^{14}CO_2$ production was measured. To determine glucose 6-phosphate levels, cells were preincubated in the presence of 10 mM-glucose for 20 min, and they were treated with 10 mM-molybdate, 5 mM-tungstate or 2 mM-vanadate for 10 min. To measure lactate production, cells were incubated for 60 min with 10 mM-glucose in the absence or in the presence of 10 mM-molybdate, 5 mM-tungstate or 2 mM-vanadate. Results are means ± S.E.M. for four independent experiments.

	$^{14}CO_2$ production (μmol of glucose/60 min per g)	Glucose 6-phosphate (nmol/g)	L-Lactate (μmol/60 min per g)
Control	0.41 ± 0.05	26 ± 4	15.1 ± 2.1
2 mM-Vanadate	1.02 ± 0.07	16 ± 2	20.0 ± 2.3
10 mM-Molybdate	1.09 ± 0.07	18 ± 2	22.2 ± 2.6
5 mM-Tungstate	1.11 ± 0.06	14 ± 3	21.1 ± 2.6

5 mM (Fig. 6). Vanadate was more potent than the other two effectors. These effects were not abolished in Ca^{2+}-depleted cells. We next studied whether molybdate and tungstate were able to counteract the decrease in Fru(2,6)P_2 levels induced by glucagon, as has been reported for vanadate [4]. Incubation of cells with 10 nM-glucagon provoked a clear decrease in Fru(2,6)P_2 levels (Fig. 5*b*). The effect of glucagon was partially counteracted when cells were incubated with 5 mM-tungstate, 10 mM-molybdate or 2 mM-vanadate (Fig. 5*b*). Therefore both molybdate and tungstate, like vanadate, were able to counteract effects of glucagon on Fru(2,6)P_2 levels.

Effects of molybdate and tungstate on PFK-2 activity

It was decided to study whether molybdate and tungstate were able to alter PFK-2 activity or, like vanadate, to counteract the inactivation of this enzyme provoked by glucagon. Neither 10 mM-molybdate nor 5 mM-tungstate was able to modify PFK-2 *a* activity (Fig. 7). Glucagon caused a clear inactivation of this enzyme. This inactivation was partially counteracted when cells were incubated in the presence of 10 mM-molybdate, 5 mM-tungstate or 2 mM-vanadate (Fig. 7). Again, molybdate and tungstate behaved like vanadate.

Effects of molybdate and tungstate on L-lactate and CO_2 production from glucose

We next examined the effect of the treatment with molybdate and tungstate on CO_2 and L-lactate production in hepatocytes incubated in the presence of 10 mM-glucose. When cells were incubated with 10 mM-molybdate or 5 mM-tungstate, a clear increase in L-lactate production was observed (Table 1). This effect was probably related to the increase in intracellular Fru(2,6)P_2 provoked by these agents. Similarly, treatment with both effectors caused a clear increase in $^{14}CO_2$ production from 10 mM-[U-^{14}C]glucose (Table 1). Likewise, in these conditions, molybdate and tungstate decreased glucose 6-phosphate levels (Table 1). These results indicate that molybdate and tungstate, like vanadate, activate glycolytic flux in isolated hepatocytes.

DISCUSSION

In this paper we show that molybdate and tungstate exert effects similar to those previously reported for vanadate in isolated rat hepatocytes [4,13]. All these three compounds have insulin-like effects on the glycolytic pathway, since they increase basal Fru(2,6)P_2 levels, counteract the effects of glucagon on Fru(2,6)P_2 concentrations and PFK-2 activity, and stimulate glycolytic flux.

On the other hand, considering glycogen metabolism, molybdate and tungstate, as previously reported for vanadate [13], provoke non-insulin-like effects, since they inactivate glycogen synthase and activate glycogen phosphorylase. In all cases the effects on glycogen synthase were produced by a mechanism independent of the presence of Ca^{2+}, since the inactivation of this enzyme was largely unaffected by depletion of intracellular Ca^{2+}. However, these three compounds activate glycogen phosphorylase through a Ca^{2+}-dependent mechanism, since the activation was lost in Ca^{2+}-depleted cells.

Regarding their effects on Fru(2,6)P_2 levels, which are independent of the presence of Ca^{2+}, vanadate is the most powerful, its effects being clearly observed at 1 mM. Tungstate requires concentrations 3–5 times higher to reach the same effect, and molybdate is about 5–10 times less effective than vanadate. The same order of relative potency was observed with respect to the inactivation of glycogen synthase and their capability to counteract glucagon effects on Fru(2,6)P_2 levels and PFK-2 activity. Regarding glycogen phosphorylase activation, vanadate is by far the most effective. Vanadate is able to produce a 6-fold activation of glycogen phosphorylase at 5 mM, whereas tungstate and molybdate were only able to produce a 2-fold activation at 20 mM. Therefore, these results indicate that vanadate is the most effective of all these three agents. The difference is even more evident when Ca^{2+}-mediated effects are taken into consideration.

Since these three compounds exert similar effects on hepatic glucose metabolism, it seems logical to assume that they act by a similar mechanism of action. A series of common biochemical properties have been described for vanadate, molybdate and tungstate, which may be responsible for the common effects observed in this paper. Thus it has been reported [29] that vanadate, molybdate and tungstate inhibited alkaline and acid phosphatases. Tungstate was always more effective than molybdate. Vanadate was least effective on acid phosphatase, but at pH 8.0 the potency of inhibition of alkaline phosphatase followed the order vanadate > tungstate > molybdate, which is exactly the same order of potency as we have observed in the present study. Probably this effect is produced because these compounds are capable of adopting structures which can be viewed as transition-state analogues for the hydrolysis of the phosphoenzyme. Since phosphorylation/dephosphorylation reactions control key steps in glycogen and glucose metabolism, the ability of these compounds to interfere with these processes may well be responsible for their effects on carbohydrate metabolism.

Their ability to act as oxidants offers another possible explanation for their common effects, since other oxidizing agents such as H_2O_2 exert powerful insulin-like effects on fat-cell glucose transport and oxidation [30,31].

Finally, another interesting possibility stems from the fact that at neutral pH these compounds form heteropolyanions containing phosphate. It has recently been reported [32] that polyoxotungstophosphates form structures which mimic, to a limited extent, the topology of the helical domains in proteins. Interactions between key regulatory proteins and these polyanions offer another speculative explanation for their biological effects.

The fact that molybdate and tungstate affect hepatocyte glucose metabolism in the same fashion as vanadate poses the problem of their effects *in vivo* when orally administered to diabetic rats. Whether molybdate and tungstate also exert normoglycaemic insulin-like effects in diabetic animals requires investigation.

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