

## Glycogenolytic, Noninsulin-like Effects of Vanadate on Rat Hepatocyte Glycogen Synthase and Phosphorylase\*

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Vanadate inactivated rat hepatocyte glycogen synthase and activated glycogen phosphorylase in a dose- and time-dependent manner. These effects were observed in hepatocytes from both fasted as well as fed rats. When rat hepatocytes were preincubated with [ $^{32}$ P]phosphate and then with vanadate, and the  $^{32}$ P-labeled glycogen synthase was specifically immunoprecipitated, it was observed that vanadate stimulated the phosphorylation of the 88,000-dalton subunit of glycogen synthase. All of the phosphate was located in the same two CNBr fragments of the enzyme which are phosphorylated by glucagon and other glycogenolytic hormones. In cells incubated in a calcium-depleted medium, vanadate was still able to inactivate glycogen synthase but its effects on phosphorylase were essentially lost. These results demonstrate that, in the hepatocyte, vanadate exerts opposite effects than in the adipocyte and skeletal muscle, where vanadate has an insulin-like action.

It has been reported that vanadate ( $\text{Na}_3\text{VO}_4$ ) exerts insulin-like actions in intact cells (see Ref. 1 for review) and has been considered an important probe to relate to insulin in studies of mechanism of action. Vanadate has been reported to stimulate *“in vitro”* tyrosine kinases (2) and be a potent and selective inhibitor of phosphotyrosyl protein phosphatases (3). Vanadate produced an insulin-like stimulation of adipocyte glucose oxidation and 2-deoxyglucose transport (4, 5). Recently, it has been demonstrated that vanadate enhances the glycogen synthase activation state in rat adipocytes similarly to insulin (6, 7). Furthermore, both vanadate and insulin increased the phosphorylation of the 95,000-dalton subunit of the adipocyte insulin receptor; this phosphorylation might be related to the intracellular activation of glycogen synthase. Stimulation of a tyrosine kinase reaction appears to be the mechanism of the vanadate-enhanced phosphorylation of the

adipocyte insulin receptor (7). Vanadate, however, differs from insulin since it does not affect phosphorylation state of serine residues of the insulin receptor. This might explain the fact that, in spite of similarities in the action of insulin and vanadate, specific differences might also exist.

In skeletal muscle vanadate increased glucose uptake, glycogen synthesis, and glycolysis, thus showing insulin-like actions, although, unlike insulin, vanadate did not change protein synthesis or degradation (8). Similarly, vanadate mimics the mitogenic action of insulin in quiescent Swiss 3T3 and 3T6 cell lines (9).

Glycogen synthase is regulated through mechanisms of phosphorylation and dephosphorylation at multiple sites (10). Phosphorylation causes the inactivation of the enzyme, whereas dephosphorylation increases its state of activation (11, 12). In rat hepatocytes insulin is able to activate glycogen synthase (13–16), whereas glucagon, epinephrine, and other glycogenolytic hormones cause a decrease in glycogen synthase activity. Glucagon exerts its effects through a cAMP-mediated mechanism, whereas epinephrine,  $\alpha$ -adrenergic agents, and vasopressin act by changing the intracellular fluxes of calcium. This inactivation is the result of the hormone-induced multiple phosphorylation of the enzyme (17–20). All phosphorylation induced by glycogenolytic hormones occurs in two cyanogen bromide (CNBr) fragments of the enzyme, denoted CB-1 and CB-2 (17–19). Glycogenolytic hormones, while inactivating glycogen synthase, provoke the activation of glycogen phosphorylase.

In the present paper we characterize the effects of vanadate on rat hepatocytes and demonstrate that, contrary to the reported insulin-like effects in rat adipocytes and skeletal muscle, in hepatocytes vanadate causes the activation of glycogen phosphorylase and the inactivation of glycogen synthase. Moreover, vanadate promotes the phosphorylation of glycogen synthase at the same regions where phosphorylation is enhanced by glucagon, epinephrine, and vasopressin.

### EXPERIMENTAL PROCEDURES

**Incubation of the Cells**—Suspensions of isolated parenchymal liver cells were prepared from starved (24 h) or, when stated, fed male Wistar rats (180–250 g) as in Ref. 21. Cells were finally resuspended in Krebs bicarbonate buffer (pH 7.4) free of glucose, pregassed with  $\text{O}_2/\text{CO}_2$  (19:1). When stated, calcium was omitted from this medium and 1 mM EGTA<sup>1</sup> was added. Aliquots ( $3.5 \text{ ml}$ ,  $4\text{--}5 \times 10^6$  cells/ml) were incubated in stoppered vials at  $37^\circ\text{C}$  with continuous shaking. At the end of the incubations, cells were centrifuged, and cell pellets were immediately homogenized with  $300 \mu\text{l}$  of ice-cold 10 mM Tris-HCl (pH 7.4) buffer containing 150 mM KF, 15 mM EDTA, 0.6 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 25  $\mu\text{g/ml}$  leupeptin, and 50 mM  $\beta$ -mercaptoethanol.

<sup>1</sup> The abbreviations used are: EGTA, [ethylenbis(oxyethylene-nitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate.

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Cell homogenates were centrifuged at  $10,000 \times g$  for 20 min at  $4^\circ\text{C}$ , and the supernatants were tested for both glycogen synthase and phosphorylase activity.

**Isolation of  $^{32}\text{P}$ -Labeled Glycogen Synthase**—Isotopic labeling of rat hepatocyte glycogen synthase was carried out by incubation of the cells ( $5\text{ ml/vial}$ ,  $4\text{--}6 \times 10^6$  cells/ml) with [ $^{32}\text{P}$ ]phosphate ( $0.1\text{ mCi/ml}$ ) in a low-phosphate ( $0.1\text{ mM}$ ) Krebs bicarbonate buffer ( $\text{pH } 7.4$ ). After 45 min exposure to [ $^{32}\text{P}$ ]phosphate, vanadate was added as required, and cells were treated as described, in order to obtain the  $10,000 \times g$  supernatants. Cytosolic supernatants containing both glycogen synthase and glycogen phosphorylase activities were prepared from the  $10,000 \times g$  fraction by ultracentrifugation at  $100,000 \times g$  for 60 min at  $4^\circ\text{C}$ .

Isolation of  $^{32}\text{P}$ -labeled glycogen synthase was carried out essentially as described in Ref. 22 using specific antibodies developed against rat liver glycogen synthase.

For CNBr cleavage of glycogen synthase, immunoprecipitates were dissolved in  $200\text{ }\mu\text{l}$  of a  $70\%$  (v/v) formic acid,  $50\text{ mM}$   $\beta$ -mercaptoethanol solution and incubated overnight at room temperature with CNBr ( $12\text{ mg/ml}$ ).

**Analytical Procedures**—Samples for SDS-polyacrylamide gel electrophoresis were evaporated to dryness and resuspended in  $30\text{ }\mu\text{l}$  of sample buffer containing  $40\text{ mM}$  Tris ( $\text{pH } 6.5$ ),  $5\%$  (w/v) SDS,  $0.6\text{ M}$   $\beta$ -mercaptoethanol,  $20\%$  (w/v) sucrose, and  $0.0125\%$  (w/v) bromophenol blue as marker. Electrophoresis in polyacrylamide gels was performed as in Ref. 23 using  $7$  or  $12.5\%$  acrylamide gels to resolve the subunit of the enzyme or the CNBr fragments, respectively.

Autoradiograms were obtained by placing the dried gels at  $-80^\circ\text{C}$  in x-ray cassettes containing films (Kodak X-Omat S) and intensifying screens. The amount of radioactivity was quantified by scanning the developed films at  $546\text{ nm}$ .

**Assay Methods**—Glycogen synthase activity ratio was measured using the low glucose-6-P/high glucose-6-P method as described in Ref. 24. Glycogen phosphorylase activity was measured as in Ref. 25. Protein was determined by the Biuret method (26) as in Ref. 27. Intracellular ATP concentration was measured enzymatically in neutralized perchloric extracts as described in Ref. 28.

**Suppliers**—[ $^{32}\text{P}$ ]Phosphate was obtained from Amersham Corp. Sodium orthovanadate was from Sigma. All other reagents were analytical grade.

## RESULTS

**Effects of Vanadate on the Activation State of Glycogen Synthase and on Glycogen Phosphorylase Activity**—When hepatocytes isolated from rats starved 24 h were incubated in the absence of glucose with increasing concentrations of vanadate for different times, a clear time- and concentration-dependent inactivation of glycogen synthase was observed. The maximal inactivation was obtained in all cases at 5 min. A concentration as low as  $0.1\text{ mM}$  vanadate already produced a significant inactivation of glycogen synthase. Larger inactivations were observed at  $1$  and  $5\text{ mM}$  (Fig. 1). Vanadate at concentrations higher than  $7\text{ mM}$  provoked the inhibition of glycogen synthase. No changes in the intracellular concentrations of ATP were observed after incubation of the cells with  $2\text{ mM}$  vanadate (or lower) for times up to 20 min. A slight decrease (about  $20\%$ ) was observed with  $5\text{ mM}$  vanadate (data not shown).

The effect of vanadate on glycogen phosphorylase activity was next examined. Incubation of the hepatocytes in the absence of glucose with increasing concentrations of vanadate for different times resulted in an increase in the glycogen phosphorylase activity. This activation was dependent on the concentration of vanadate and the time of incubation. Maximal effects were obtained at 3 min of incubation. At the highest concentration of vanadate tested ( $5\text{ mM}$ ), glycogen phosphorylase activity was increased 6-fold over the control values (Fig. 2). However, the effect of vanadate on glycogen synthase was stable with time, whereas the effect on glycogen phosphorylase activity was transient. These results indicate that in hepatocytes vanadate exerts effects opposite to those

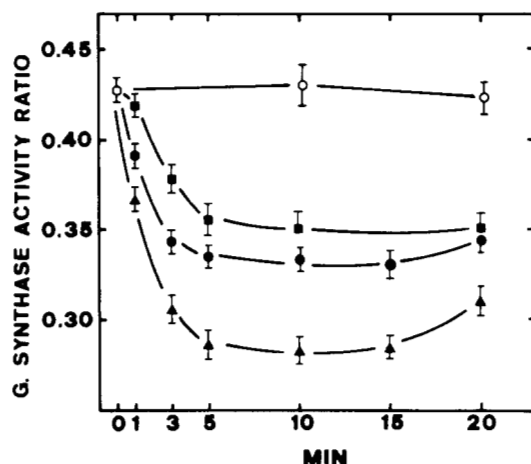


FIG. 1. Time- and dose-dependent effects of vanadate on glycogen synthase activity ratio. Aliquots of the cells were incubated without (○) or with  $0.1$  (■),  $1$  (●), and  $5\text{ mM}$  (▲) vanadate for the indicated times. At the end of the incubations, the cells were homogenized and glycogen synthase low ( $0.25\text{ mM}$ )/high ( $10\text{ mM}$ ) glucose-6-P activity ratio was measured. Results are means  $\pm$  S.E. of at least five independent experiments.

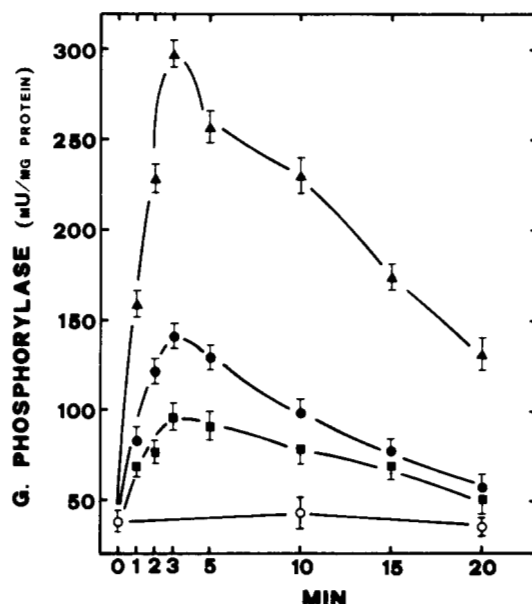


FIG. 2. Time- and dose-dependent effect of vanadate on glycogen phosphorylase activity. Hepatocytes were incubated without (○) or with  $0.1$  (■),  $1$  (●), and  $5\text{ mM}$  (▲) vanadate for different times. Extracts were then prepared and glycogen phosphorylase  $a$  activity was measured as indicated. Results are means  $\pm$  S.E. of at least five independent experiments.

of insulin and similar to those provoked by glycogenolytic hormones.

**Effects of Vanadate on the Phosphorylation State of Glycogen Synthase**—In order to study whether the inactivation produced by vanadate was a consequence of the phosphorylation of the enzyme, hepatocytes were incubated with [ $^{32}\text{P}$ ]phosphate and then with vanadate. Glycogen synthase was immunoprecipitated from both control and vanadate-treated cells, and the immunoprecipitates were submitted to polyacrylamide gel electrophoresis in the presence of SDS, and then autoradiograms were obtained (Fig. 3A).

Incubation of the hepatocytes with  $2\text{ mM}$  vanadate for 5 min provoked an important increase in the amount of  $^{32}\text{P}$  bound to the  $88,000$ -dalton subunit of the enzyme, indicating that the inactivation observed was probably related to the

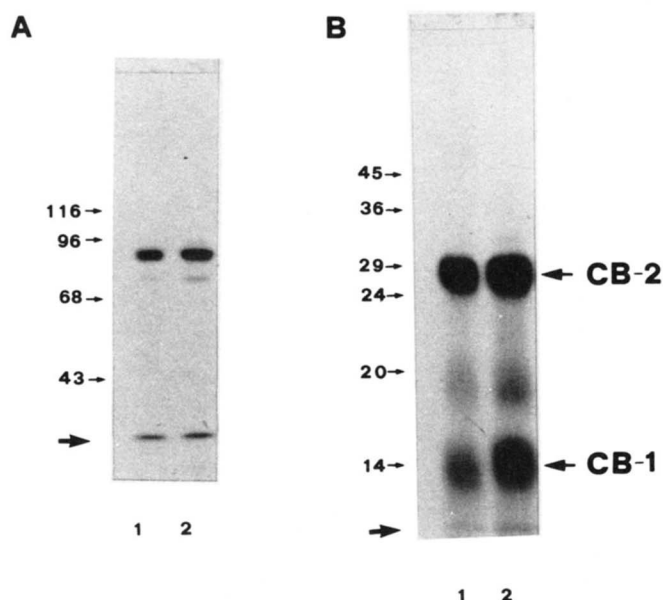


FIG. 3. Electrophoretic analysis of  $^{32}\text{P}$ -labeled glycogen synthase from rat hepatocytes.  $^{32}\text{P}$ -Labeled glycogen synthase was immunoprecipitated from [ $^{32}\text{P}$ ]phosphate-preincubated cells. The immunoprecipitates were subjected to polyacrylamide gel electrophoresis before (A) or after (B) CNBr cleavage. Lanes 1A and 1B are autoradiograms of immunoprecipitates from control cells; lanes 2A and 2B, immunoprecipitates from cells exposed to 2 mM vanadate for 5 min. Conditions for electrophoresis are given in the text. Molecular weights (in thousands) of standard proteins are shown on the left.

TABLE I

Effects of vanadate on the phosphorylation state of glycogen synthase

$^{32}\text{P}$ -glycogen synthase from control or vanadate-treated cells was immunoprecipitated. Electrophoresis were performed before or after CNBr treatment. Autoradiograms of gels were scanned, and the peaks were integrated. Results are expressed as a percentage of the values of the peaks from control cells and are means  $\pm$  S.E. of at least five independent experiments.

	Glycogen synthase $^{32}\text{P}$ subunit	$^{32}\text{P}$ -CNBr fragments		
		CB-2	CB-1	CB-2/CB-1
Control	100	100	100	$3.14 \pm 0.31$
Vanadate (2 mM)	$217 \pm 21$	$159 \pm 12$	$237 \pm 26$	$1.57 \pm 0.21$

phosphorylation of glycogen synthase.

To obtain more insight into the phosphorylation of glycogen synthase induced by vanadate, immunoprecipitates were cleaved with CNBr, and the resulting phosphopeptides were resolved by electrophoretic analysis. Autoradiograms showed two  $^{32}\text{P}$ -containing fragments, CB-2 ( $M_{\text{app}}$  28,000) and CB-1 ( $M_{\text{app}}$  14,000) (Fig. 3B).

Vanadate affected the phosphorylation state of both CNBr fragments. When compared to control, radioactivity in CB-2 was increased by a factor of 1.6 and radioactivity present in CB-1 increased at least 2-fold. These results indicated that vanadate stimulates the phosphorylation of glycogen synthase at multiple sites (Table I).

**Effects of Vanadate on Glycogen Synthase and Glycogen Phosphorylase in Calcium-depleted Cells**—To investigate the possible role of calcium in the mechanism of action of vanadate we next examined whether the effects of vanadate were modified when calcium ions were removed from the incubation medium. Therefore, cells were incubated in the absence of glucose in a normal Krebs-Ringer medium and in a calcium-depleted medium containing 1 mM EGTA. In the absence of calcium, cells showed a higher glycogen synthase activity ratio and slightly lower phosphorylase  $\alpha$  activity. Incubation of the

cells for 5 min with increasing concentrations of vanadate resulted in parallel decreases in glycogen synthase activity ratio in the presence or absence of calcium (Fig. 4A). Similarly, incubation with 1 mM vanadate showed, again, parallel time courses of inactivation with or without calcium (Fig. 4B).

In contrast, depletion of extracellular calcium almost completely abolished the activation of glycogen phosphorylase induced by vanadate (Fig. 4C). Only at a high concentration of vanadate (5 mM) was a small increase in the activity of the enzyme observed. No effects on glycogen phosphorylase activity were observed in calcium-depleted cells at concentrations of vanadate 1 mM or below (Fig. 4D). These results suggest that calcium is involved in the mechanism by which vanadate activates glycogen phosphorylase, whereas the effects on glycogen synthase are independent of this cation.

**Effects of Vanadate on Glycogen Synthase and Glycogen Phosphorylase Activities on Fed Rats**—Since the effects of vanadate could be influenced by the nutritional state of the animals, we studied the response of glycogen synthase and glycogen phosphorylase in hepatocytes isolated from fed rats. As shown in Fig. 5, vanadate was still able to inactivate glycogen synthase and activate glycogen phosphorylase in these cells, although the responses were less marked than in fasted animals. At a concentration of 5 mM vanadate, glycogen phosphorylase was only activated 3-fold, changes in the glycogen synthase activity ratio were less pronounced, and max-

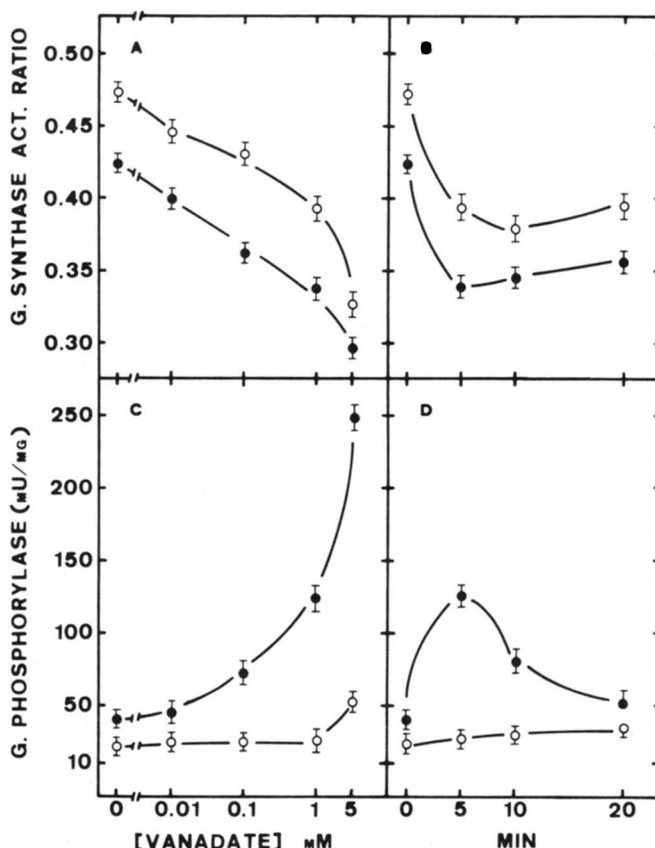


FIG. 4. Calcium dependence of vanadate effects on glycogen synthase and glycogen phosphorylase activities in rat hepatocytes. Cells incubated in normal (●) or calcium-depleted Krebs-Ringer medium (○) were incubated with vanadate. Then glycogen synthase low glucose-6-P/high glucose-6-P activity ratio and phosphorylase  $\alpha$  activity were measured. Conditions are: A and C, hepatocytes incubated with vanadate for 5 min at different concentrations; B and D, cells incubated with 1 mM vanadate for the indicated times. Results are means  $\pm$  S.E. of at least four independent experiments.



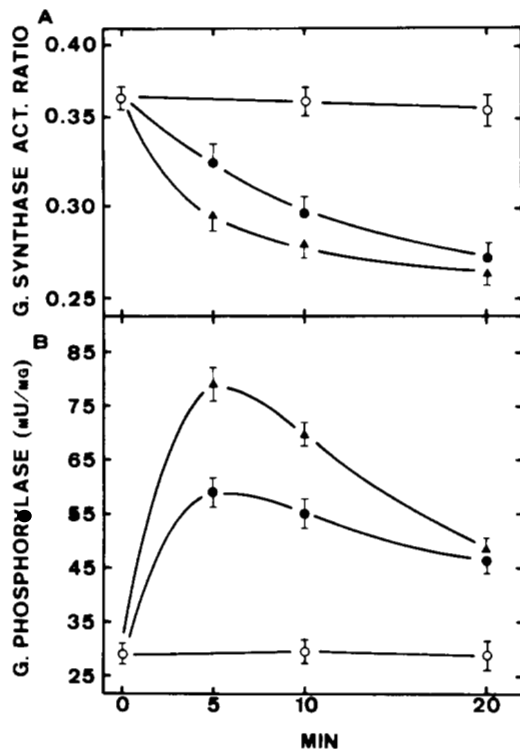


FIG. 5. Effects of vanadate on glycogen synthase and glycogen phosphorylase activities of hepatocytes from fed rats. Hepatocytes were prepared from fed rats and incubated without (○) or with 1 (●) or 5 mM (▲) vanadate for different times. Glycogen synthase activity ratio (A) and glycogen phosphorylase  $\alpha$  activity (B) were measured. Results are means  $\pm$  S.E. of at least three independent experiments.

imal effects were obtained at longer times of incubation with the effector.

In any case, although quantitatively the responses were different, qualitatively the effects of vanadate were maintained in cells from fed rats.

#### DISCUSSION

The results presented in this paper indicate that in rat hepatocytes vanadate acts oppositely to insulin. In these cells vanadate activates glycogen phosphorylase and inactivates glycogen synthase in a concentration- and time-dependent manner. These effects are exactly opposite to those of an insulin-like agent. Therefore, the effects of vanadate in rat hepatocytes completely differ from those observed in rat adipocytes (6, 7) or diaphragm where this compound causes an activation of glycogen synthase, thus showing insulin-like action.

The inactivation of glycogen synthase is the result of the phosphorylation of the enzyme as probed in the experiments using cells incubated with [ $^{32}\text{P}$ ]phosphate. We have used antibodies raised in rabbit against rat liver glycogen synthase to immunoprecipitate the enzyme. At the concentration used the antibodies were effective in completely removing glycogen synthase activity from extracts (17). This immunoprecipitate was highly specific, since the 88,000-dalton band, corresponding to the subunit of glycogen synthase, was the only radioactive band obtained. However, maximal care had to be taken to avoid proteolysis of the enzyme, since a proteolytic band of 80,000 daltons could easily appear.

Glycogen synthase is phosphorylated by the action of vanadate into two CNBr fragments, CB-1 and CB-2. These are precisely the same peptides which are phosphorylated as a

result of the incubation of the hepatocytes with glycogenolytic hormones (17–19). Since both fragments increase their phosphate content in response to vanadate, it must be concluded that vanadate stimulates the phosphorylation of the enzyme at multiple sites. Although more phosphate was actually introduced in the largest CNBr fragment, CB-2, on a percentage basis the increase in the radioactivity in the smaller CNBr fragment, CB-1, was more pronounced, indicating that the sites present in CB-1 are preferentially phosphorylated by incubation of the cells with vanadate. Again, this is the phosphopeptide which, on a percentage basis, is mainly affected by incubation with glycogenolytic hormones and other effectors under the same experimental conditions (17, 22).

In conclusion, our results show that, in liver, vanadate does not show insulin-like activity. On the contrary, its action is reminiscent of the glycogenolytic hormones. Therefore, although vanadate has been so far considered to be an insulin-like agent, because of its insulin-like effects on adipocytes and skeletal muscle, our results indicate that this behavior cannot be extrapolated to all tissues since, at least in hepatocytes, the effects of vanadate on glycogen synthase and phosphorylase are clearly opposite to those of insulin.

It has been recently reported that vanadate, when administered to diabetic rats, reduces the high blood glucose levels present in these animals (29). Our findings suggest that these "in vivo" results must be only due to an increase in the uptake and utilization of glucose by adipocytes and skeletal muscle, since our results do not favor the hypothesis that vanadate reduces the hepatic glucose output. In this regard, it must be stressed that the glycogenolytic hormone-like effects of vanadate have also been observed in hepatocytes from fed animals, indicating that the pattern of action of vanadate is independent of the nutritional state of the animal.

With regard to the mechanism of action of vanadate, our results suggest that it acts through different mechanisms in the activation of glycogen phosphorylase and in the inactivation of glycogen synthase. It can be concluded that the effects of vanadate on glycogen phosphorylase are dependent on a calcium mediated mechanism, whereas those on glycogen synthase are independent of external calcium. This dissociation of effects in the absence of calcium has not been observed with any other hormone or glycogenolytic effector so far studied. Therefore, vanadate might be a useful tool in the study of the mechanisms which coordinate the inverse regulation of the two key enzymes of glycogen metabolism in the liver. Since vanadate has been shown to activate kinases (2, 7, 30) and inhibit phosphatases (3), both mechanisms could explain the observed increased phosphorylation of glycogen synthase. A more detailed analysis of the sites of glycogen synthase phosphorylated by vanadate, which is presently being carried out in our laboratory, might contribute to the understanding of the regulatory mechanisms affected by vanadate.

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