Biochem. J. (1988) 255, 507–512 (Printed in Great Britain)

Vanadate raises fructose 2,6-bisphosphate concentrations and activates glycolysis in rat hepatocytes

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In rat hepatocytes, vanadate increases fructose 2,6-bisphosphate (Fru-2,6-P_2) in a time- and dose-dependent manner, and counteracts the decrease in this metabolite caused by glucagon, forskolin or exogenous cyclic AMP. Vanadate does not directly modify the activity of 6-phosphofructo-2-kinase, even though it can counteract the inactivation of this enzyme caused by glucagon. Furthermore, vanadate raises the yield of ^3H_2O from [3-^{3}H]glucose, indicating that it increases the flux through 6-phosphofructo-1-kinase. Moreover, vanadate in hepatocytes incubated in the presence of glucose increases the production of both lactate and CO_2. Therefore vanadate has insulin-like effects on the glycolytic pathway in rat hepatoyctes. These results clearly contrast with our previous observation that vanadate exerts glycogenolytic non-insulin-like effects on glycogen synthase and phosphorylase.

INTRODUCTION

In several tissues vanadate shows insulin-like actions, activating glucose transport and oxidation [1–3]. In addition, several reports indicate that the oral administration of vanadate to diabetic rats normalizes blood glucose concentrations and also appears to restore insulin-responsiveness of target tissues [4–6]. However, we have recently demonstrated [7] that vanadate exerts a non-insulin-like effect on glycogen-metabolizing enzymes in rat hepatocytes. In these cells vanadate activates glycogen phosphorylase, and phosphorylates and inactivates glycogen synthase, more closely resembling the behaviour of the glycogenolytic hormones. It was therefore decided to see whether these non-insulin-like effects of vanadate in rat hepatocytes were limited to glycogen metabolism, or if they extended to the glycolytic/gluconeogenic pathway.

Fru-2,6-P_2 is the metabolite that exerts major control of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in the liver. Glucagon and β-adrenergic agents that increase cyclic AMP control the concentration of Fru-2,6-P_2. They activate cyclic AMP-dependent protein kinase, which regulates the activity of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (for a review see [8]). Although insulin is not able to increase basal concentrations of Fru-2,6-P_2, it counteracts the actions of glucagon or β-adrenergic agonists on Fru-2,6-P_2 by decreasing cyclic AMP, probably through an activation of cyclic AMP phosphodiesterase. This action of insulin results in less marked decreases in Fru-2,6-P_2 induced by these agents [9,10]. On the other hand, glycogenolytic hormones which act through a Ca^{2+}-mediated mechanism increase Fru-2,6-P_2 in hepatocytes from fed, but not from starved, rats. Their effect probably results from an increased concentration of hexose 6-phosphates [11].

In the present paper we have measured the effects of vanadate on Fru-2,6-P_2 concentrations and on the activity of 6-phosphofructo-2-kinase. In addition, the production of ^3H_2O from [3-^{3}H]glucose was determined to evaluate the flux through 6-phosphofructo-1-kinase. Lacate and CO_2 production have been measured to estimate the extent of glycolysis and glucose oxidation respectively. The conclusion of this study is that vanadate exerts insulin-like effects on the glycolytic/gluconeogenic pathway. These results clearly contrast with our previous observation that vanadate exerts glycogenolytic non-insulin-like effects on glycogen synthase and phosphorylase.

EXPERIMENTAL

Preparation and incubation of hepatocytes

Suspensions of isolated parenchymal liver cells were prepared from 24 h-starved or, when stated, fed male Wistar rats (180–250 g) as in ref. [12]. Cells were finally resuspended in Krebs bicarbonate buffer (pH 7.4), pregassed with O_2/CO_2 (19:1). When stated, Ca^{2+} was omitted from this medium and 1 mM-EGTA was added. When Fru-2,6-P_2 was to be determined, cells were resuspended in a lactate/pyruvate (16 mM/4 mM)-containing Krebs bicarbonate buffer (pH 7.4). Portions [(4–5) × 10^6 cells/ml] were incubated in stoppered vials at 37 °C with continuous shaking.

Radiochemical determination of flux through 6-phosphofructo-1-kinase

The flux through 6-phosphofructo-1-kinase was estimated from the rate of ^3H_2O formation from [3-^{3}H]glucose. Samples (3 ml) of cell suspension were incubated for 60 min in the presence of 10 mM-[3-^{3}H]glucose (30 μCi/mmol). After centrifugation at 3000 rev./min for 1 min, supernatants were applied to columns of Dowex AG1 (X8; borate form) to separate ^3H_2O from [3-^{3}H]glucose [13].

Abbreviation used: Fru-2,6-P_2, fructose 2,6-bisphosphate.

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Enzyme and metabolite assays

For measurement of Fru-2,6-P_2 concentrations and 6-phosphofructo-2-kinase activity, 0.1 ml samples of cell suspension were frozen in liquid N_2. To determine Fru-2,6-P_2, frozen samples were thawed in 0.6 ml of 54 mM-NaOH and, after heating at 80 °C for 5 min, Fru-2,6-P_2 was measured by its ability to activate PP_i:fructose-6-phosphate 1-phosphotransferase, by the method described by Van Schaftingen *et al.* [14]. To determine the activity of 6-phosphofructo-2-kinase, frozen samples were thawed in 0.1 ml of a buffer containing 20 mM-potassium phosphate, 10 mM-EDTA and 100 mM-KCl, pH 7.0. After centrifugation (12000 *g* for 10 min at 4 °C), 6-phosphofructo-2-kinase activity of the resulting supernatants was determined as described by Bartrons *et al.* [15], by the enzyme assay for the 'active' form at pH 6.6. To determine glucose 6-phosphate, 2 ml samples of hepatocyte suspension were centrifuged and cell pellets were immediately homogenized with 0.5 ml of ice-cold 10% (w/v) $HClO_4$. Glucose 6-phosphate concentration was measured enzymically in neutralized $HClO_4$ extracts. Glucose and L-lactate concentrations were measured enzymically in the 12000 *g* cell supernatants.

To determine $^{14}CO_2$ production, hepatocytes (3 ml) were incubated with 10 mM-[U-^{14}C]glucose. At the end of the incubation period, a piece of filter paper placed into the specially adapted vials was soaked with 0.2 ml of β-phenethylamine. After addition of 0.5 ml of 10% $HClO_4$ to the cell suspension, the vials were shaken for another 30 min, and then filter papers were counted for radioactivity.

Materials

Sodium orthovanadate, glucagon, cyclic AMP and [U-^{14}C]glucose were from Sigma. [3-^{3}H]Glucose was from Amersham International. Forskolin was from Calbiochem.

RESULTS

Effects of vanadate on Fru-2,6-P_2 intracellular concentrations

Hepatocytes obtained from starved rats were preincubated for 30 min in a Krebs–Ringer medium in the absence of glucose but containing 16 mM-lactate/4 mM-pyruvate, to maintain basal intracellular Fru-2,6-P_2 concentrations. Fig. 1 shows that incubation of hepatocytes with 2 mM-vanadate for different times resulted in a time-dependent increase in intracellular Fru-2,6-P_2. This effect was maximal after incubation for 5 min. The effect of vanadate was also concentration-dependent (Fig. 2). It was already significant at 0.1 mM-vanadate; at 5 mM-vanadate Fru-2,6-P_2 was increased approx. 1.9-fold. Doses higher than 5 mM were not used, since they produce a decrease in intracellular ATP [7]. Hepatocytes incubated in a glucose-free Krebs–Ringer medium without addition of lactate/pyruvate had a very low basal concentration of intracellular Fru-2,6-P_2. In these cells 5 mM-vanadate produced a greater increase (8-fold) in this metabolite (results not shown).

Since in a previous paper [7] we showed that some of the effects of vanadate were abolished in Ca^{2+}-depleted cells, we next studied whether the effect of vanadate on Fru-2,6-P_2 was dependent on the presence of Ca^{2+}. As shown in Fig. 2, in hepatocytes incubated in the absence

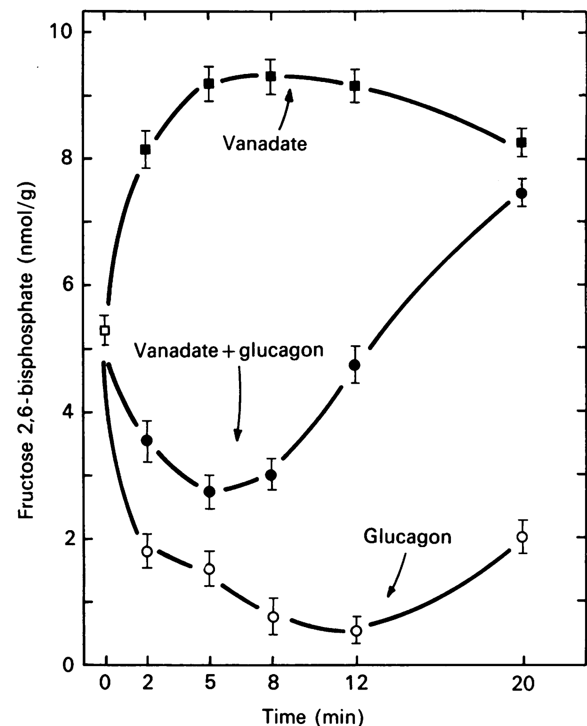

Fig. 1. Time course of vanadate effects on Fru-2,6-P_2 concentrations

Cells were preincubated in Krebs–Ringer medium with 16 mM-lactate/4 mM-pyruvate for 30 min. Then hepatocytes were exposed for the indicated times to 2 mM-vanadate, 10 nM-glucagon or 2 mM-vanadate plus 10 nM-glucagon. Values are means ± S.E.M. for six experiments performed on different days.

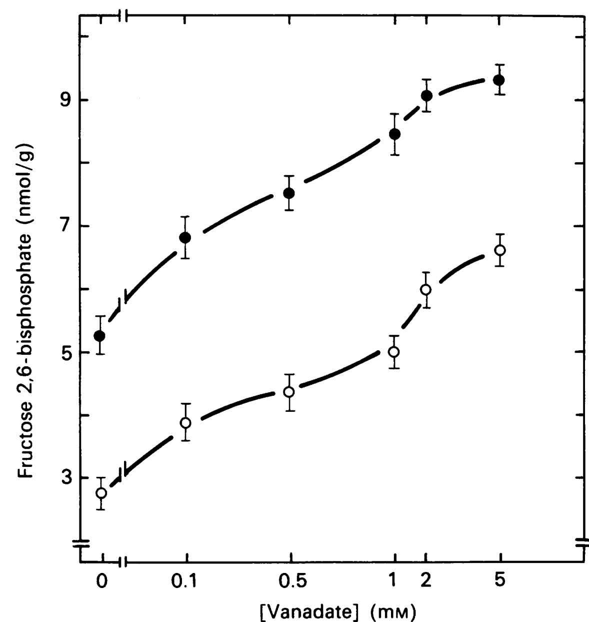

Fig. 2. Dose-dependence of the effect of vanadate on Fru-2,6-P_2 in normal or Ca^{2+}-depleted cells

Hepatocytes from starved rats were preincubated for 30 min in normal (●) or Ca^{2+}-depleted (○) Krebs–Ringer medium in the presence of 16 mM-lactate/4 mM-pyruvate, and then treated for 5 min with different concentrations of vanadate. Values shown are means ± S.E.M. for four independent experiments.

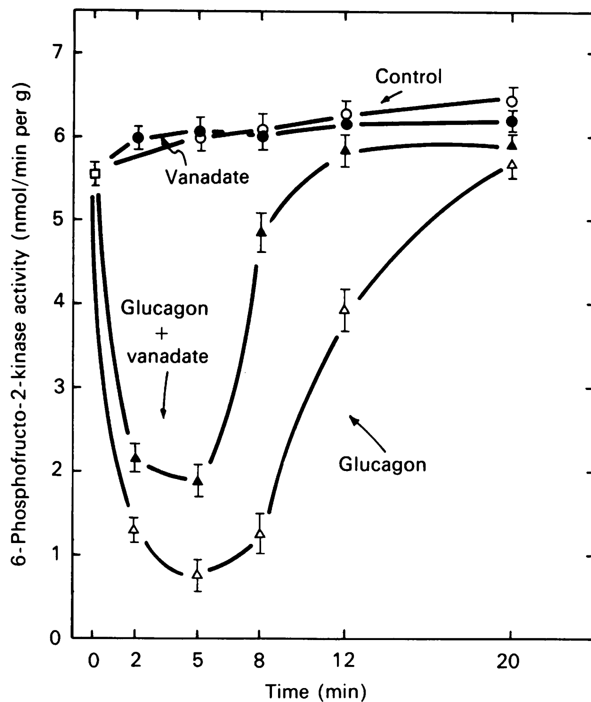

Fig. 3. Effects of vanadate on 6-phosphofructo-2-kinase activity

Hepatocytes from starved rats were preincubated for 30 min in Krebs–Ringer medium containing 16 mM-lactate/4 mM-pyruvate. Then hepatocytes were treated for different times with 2 mM-vanadate, 10 nM-glucagon or 2 mM-vanadate plus 10 nM-glucagon. Results are means ±S.E.M. for four different experiments.

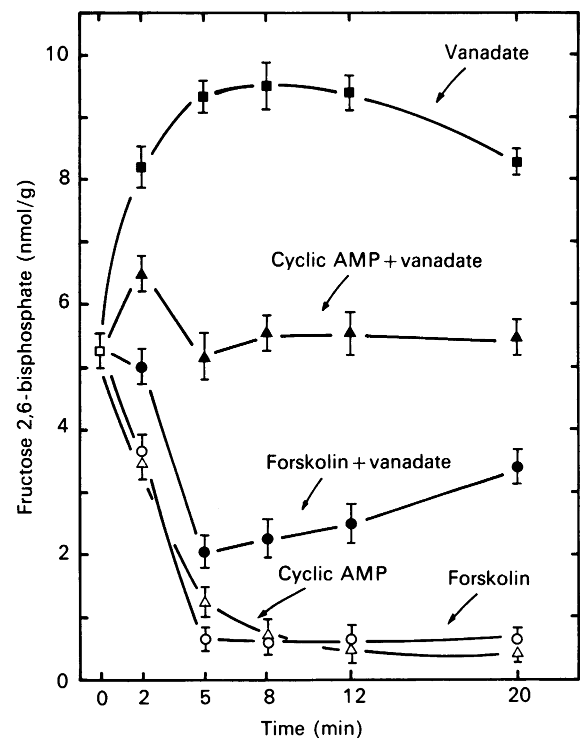

Fig. 4. Time course of the effects of vanadate on decreases in Fru-2,6-P_2 induced by exogenous cyclic AMP or forskolin

Isolated hepatocytes were incubated for 30 min in Krebs–Ringer medium containing 16 mM-lactate/4 mM-pyruvate. Then cells were treated, for the indicated times, with 2 mM-vanadate plus either 0.1 mM exogenous cyclic AMP or 0.1 mM-forskolin. Results are means ± S.E.M. for four independent experiments.

of Ca^{2+} the basal concentrations of Fru-2,6-P_2 were decreased as compared with cells incubated in normal medium. However, in both cases, vanadate caused parallel increases in Fru-2,6-P_2, indicating that this action of vanadate is not mediated by Ca^{2+}.

Effects of vanadate on 6-phosphofructo-2-kinase activity

We next studied the mechanism by which vanadate increased basal concentrations of Fru-2,6-P_2. Since we have recently shown that vanadate does not modify basal cyclic AMP concentrations or cyclic AMP-dependent protein kinase activity [16], it was then necessary to determine whether vanadate directly activates 6-phosphofructo-2-kinase. As shown in Fig. 3, the activity of this enzyme was not affected by the incubation of hepatocytes with 2 mM-vanadate for different times.

Counteraction by vanadate of the effects of glucagon

The effect of vanadate on Fru-2,6-P_2 was opposite to that reported for glucagon [17,18]. Therefore we investigated the combined effects of vanadate and glucagon. Glucagon alone at 10 nM provoked a decrease to 14% of control in the concentration of this metabolite. Incubation of the cells with both vanadate and glucagon resulted in a counteraction of the effects of glucagon by vanadate (Fig. 1).

The next series of experiments were undertaken to investigate the mechanism by which vanadate counteracts the effects of glucagon. The counteracting effects of vanadate could be exerted either on or before adenylate cyclase, or on cyclic AMP phosphodiesterase. Therefore vanadate was tested against 0.1 mM-forskolin, which activates adenylate cyclase by a non-receptor-mediated mechanism [19]. Vanadate counteracted the decrease in the metabolite provoked by forskolin, suggesting that its action has to be exerted at a post-receptor level (Fig. 4). Fig. 4 also shows that vanadate lessened the decrease in Fru-2,6-P_2 induced by 0.1 mM exogenous cyclic AMP, indicating that its action is exerted at a post-adenylate cyclase step. Therefore the most probable alternative is that vanadate increases phosphodiesterase activity. In fact, we have recently observed that, although vanadate does not modify basal concentrations of cyclic AMP, it counteracts the increase in this metabolite induced by glucagon (C. Villar-Palasi, A. M. Gómez-Foix, J. E. Rodríguez-Gil, J. J. Guinovart & F. Bosch, unpublished work). As could be expected from the results presented above, vanadate also counteracted the inactivating effects of glucagon on 6-phosphofructo-2-kinase (Fig. 3).

Effects of vanadate on the glycolytic flux

In order to see whether the increase in Fru-2,6-P_2 had an immediate effect on the flux through the glycolytic pathway, we measured the flux through 6-phosphofructo-1-kinase as the release of ^3H_2O from [3-^{3}H]glucose. In hepatocytes from starved rats, 2 mM-vanadate clearly increased the detritiation of [3-^{3}H]glucose in a concentration-dependent manner (Fig. 5), indicating

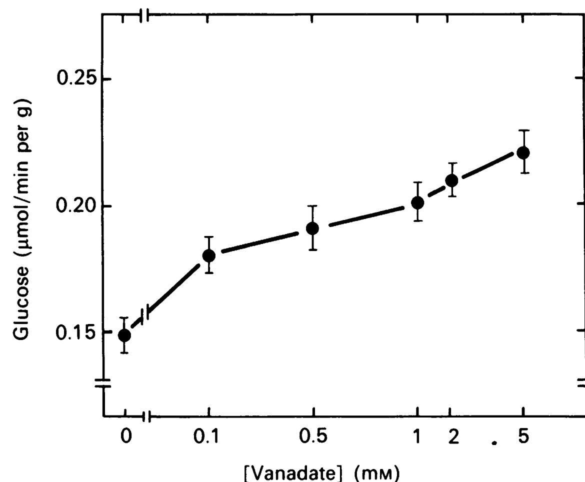

Fig. 5. Effect of vanadate on the flux through 6-phosphofructo-1-kinase

Hepatocytes from starved rats were incubated for 60 min with 10 mM-[3-^{3}H]glucose in the presence of different concentrations of vanadate. At the end of the incubations ^3H_2O released was measured. Results shown are means ± S.E.M. for four different experiments.

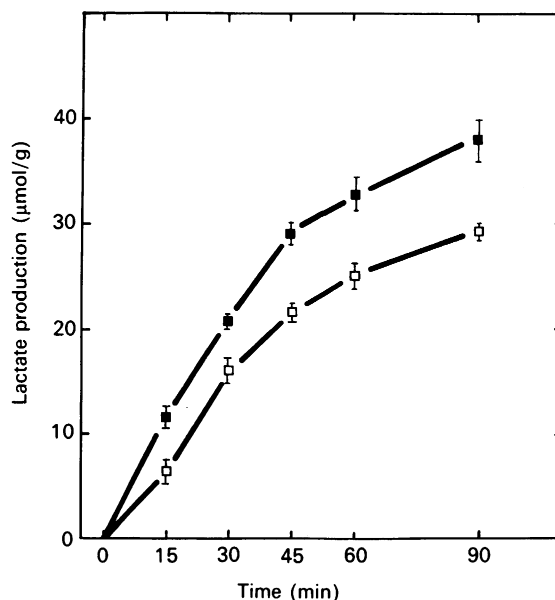

Fig. 6. Time course of the vanadate action on L-lactate production

Hepatocytes from starved rats were incubated with 15 mM-glucose in the absence (□) or in the presence (■) of 2 mM-vanadate. Values are means ± S.E.M. for three cell preparations.

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

Hepatocytes obtained from starved rats were preincubated for 30 min with 16 mM-lactate/4 mM-pyruvate and further treated for 10 min with 2 mM-vanadate to determine glucose 6-phosphate. Hepatocytes were incubated for 60 min with 10 mM-[U-^{14}C]glucose in the absence or presence of 2 mM-vanadate to measure $^{14}CO_2$ production. Cells were incubated for 60 min with 15 mM-glucose in the absence or presence of 2 mM-vanadate to determine glucose uptake. Values are means ± S.E.M. for four different experiments.

	Glucose 6-phosphate (nmol/g)	$^{14}CO_2$ production (µmol of glucose oxidized/ 60 min per g)	Glucose uptake (µmol/ 60 min per g)
Control	31 ± 2	0.46 ± 0.05	14 ± 1
+ Vanadate	20 ± 3	1.01 ± 0.08	27 ± 3

that vanadate increases the flux through 6-phospho-fructo-1-kinase. Hence, glucose 6-phosphate was decreased in vanadate-stimulated cells under conditions where Fru-2,6-P_2 was increased (Table 1). To determine the fate of glucose, we also measured the production of lactate in hepatocytes from starved rats incubated with 15 mM-glucose for different periods of time. As shown in Fig. 6, 2 mM-vanadate caused a significant increase in the production of lactate. Similarly the oxidation of glucose, measured by the amount of $^{14}CO_2$ released from 10 mM-[U-^{14}C]glucose, was doubled by vanadate (Table 1). Therefore vanadate provoked an increase in both the production of lactate and the oxidation of glucose. Simultaneously, vanadate stimulated the uptake of glucose from the medium (Table 1).

Similar experiments were performed in hepatocytes from fed rats incubated in the absence of glucose, to measure the fate of glucose released from glycogen by vanadate. In these cells vanadate increased the production of lactate, from 11 ± 2 to 23 ± 3 µmol/30 min per g, but did not affect the amount of glucose released into the medium (33 ± 2 for control versus 34 ± 3 µmol/ 30 min per g for vanadate-treated cells).

DISCUSSION

In the present paper we show that in rat hepatocytes vanadate exerts insulin-like effects on glycolysis. We must recall that vanadate exerts glycogenolytic, non-insulin-like, effects on rat hepatocyte glycogen synthase and phosphorylase [7]. Therefore, a first conclusion from this work is that in the same tissue vanadate may exert insulin or non-insulin-like effects, depending on the pathway studied.

We have shown that vanadate increases in a time- and concentration-dependent manner Fru-2,6-P_2, the most important regulatory metabolite of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle. This effect of vanadate is unique. No other known effector (other than glucose or fructose [11,20]) can increase Fru-2,6-P_2 in hepatocytes from fasted rats to the concentration produced by vanadate. α-Adrenergic agents and vasopressin increase Fru-2,6-P_2 in hepatocytes from fed, but not from fasted, rats [9,11]. The effect of these glycogenolytic hormones has been attributed to a 'push' effect, owing to the increase in glucose 6-phosphate [11]. Insulin is not able to raise the basal concentration of this metabolite. Glucagon is the most powerful agent in causing a decrease in Fru-2,6-P_2. Therefore the effects of vanadate, with regard to modifying Fru-2,6-P_2 concentrations, are clearly opposite to those of glucagon. It is worth noting that, as regards their actions on glycogen-metabolizing enzymes, glucagon and vanadate exert similar effects [7].

The mechanism by which vanadate modifies basal concentrations of Fru-2,6-P_2 has also been studied. We have shown that vanadate activates glycogen phosphorylase through a Ca^{2+}-dependent mechanism, whereas its effects on glycogen synthase are independent of Ca^{2+}. Therefore we first checked whether vanadate required Ca^{2+} to affect Fru-2,6-P_2. Since the action of vanadate on this metabolite is not abolished in Ca^{2+}-depleted cells, we must conclude that it is not mediated by Ca^{2+}. 6-Phosphofructo-2-kinase, the enzyme responsible for the formation of Fru-2,6-P_2, is inactivated by cyclic AMP-dependent protein kinase. Therefore a decrease in cyclic AMP could be an explanation for the effects of vanadate. However, in rat hepatocytes vanadate does not change either the basal concentration of cyclic AMP or the activation state of cyclic AMP-dependent protein kinase [16]. Thus a decrease in cyclic AMP is not the explanation for the effects of the agent. Another possible mechanism would be an increase in glucose 6-phosphate, which would augment substrate availability for 6-phosphofructo-2-kinase. However, vanadate is able to increase Fru-2,6-P_2 8-fold in hepatocytes from fasted rats incubated in the absence of glucose or gluconeogenic precursors. Furthermore, vanadate causes a decrease rather than an increase in intracellular glucose 6-phosphate in hepatocytes from fasted rats incubated with lactate/pyruvate, under conditions where Fru-2,6-P_2 was increased.

Finally, we measured the activity of 6-phosphofructo-2-kinase in extracts from hepatocytes treated with vanadate. No effects were found. Therefore, vanadate does not provoke a stable activation of this enzyme. A similar effect was observed in hepatocytes incubated with chlorpropamide [21]. It is also possible that 6-phosphofructo-2-kinase has a second mechanism of activation, the effect of which is lost in the extracts, in addition to the permanent, well-characterized, mechanism of phosphorylation/dephosphorylation of the bifunctional enzyme. Therefore we are tempted to speculate that vanadate, directly or indirectly, causes a non-covalent activation of 6-phosphofructo-2-kinase.

Although insulin is not able to increase basal concentrations of Fru-2,6-P_2, it can counteract the decrease provoked by glucagon. Vanadate shares this action with insulin, since it effectively counteracts the effects of glucagon. This effect of vanadate is probably due to its capacity to diminish the increase in cyclic AMP induced by glucagon and the resulting activation of the cyclic AMP-dependent protein kinase (C. Villar-Palasi, A. M. Gómez-Foix, J. E. Rodríguez-Gil, J. J. Guinovart & F. Bosch, unpublished work). Since vanadate is also able to counteract the effects of forskolin and of exogenous cyclic AMP, we must conclude that this action of vanadate is exerted through phosphodiesterase. In this regard vanadate perfectly mimics the action of insulin. Altogether, vanadate appears to have two mechanisms of action on Fru-2,6-P_2 concentrations, one direct and independent of cyclic AMP, and a second one based on its ability to counteract cyclic AMP-mediated effects. This behaviour is similar to that of insulin on glycogen synthase. The hormone activates this enzyme through a mechanism independent of cyclic AMP, but is also able to counteract the effects of those hormones acting through an increase in cyclic AMP.

The increase in Fru-2,6-P_2 clearly results in an increase in the flux through the glycolytic pathway as measured by the detritiation of [3-^{3}H]glucose. As a consequence, both the production of lactate and the oxidation of glucose are increased. Simultaneously, the uptake of glucose is stimulated by vanadate. Therefore, vanadate activates both glycolysis and glycogenolysis. We can therefore imagine that the glucose moieties removed from glycogen are immediately shuffled into glycolysis and diverted from being released to the medium. In fact, this is what is observed when vanadate is added to hepatocytes from fed rats: in these conditions the release of glucose is not affected, whereas the production of lactate is increased.

The results described in this paper support the concept that the ability of vanadate to activate hepatic glucose consumption, through an increase in Fru-2,6-P_2, contributes to the observed capacity of vanadate to normalize glycaemia when administered orally to diabetic rats [4–6], a mechanism that could be shared with chlorpropamide and tolbutamide [21,22], two widely used oral hypoglycaemic agents.

This work was supported by a grant from the 'Comisión Asesora a la Investigación Científica y Técnica' (PB 85-0159), Spain. A. M. G.-F. was recipient of a grant from the CIRIT (Barcelona, Spain), and J. E. R.-G. was recipient of a Fellowship from the F.P.I. (Spain). We thank Dr. C. Villar-Palasí and Dr. Anna Bassols for helpful suggestions, and Anna Vilalta and Catalina Relaño for their skilled technical assistance.

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Received 15 February 1988/19 May 1988; accepted 31 May 1988