Permolybdate and Pertungstate—Potent Stimulators of Insulin Effects in Rat Adipocytes: Mechanism of Action†

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ABSTRACT: In previous studies, tungstate and molybdate were found to mimic the biological actions of insulin. It was suggested that these metallooxides initially inhibit vanadate-sensitive protein phosphotyrosine phosphatase (PTPase). This, in turn, stimulates a staurosporine-sensitive cytosolic protein tyrosine kinase (cytPTK), which activates several insulin bioeffects via insulin-independent pathways (Shisheva & Shechter, 1991, 1993; Elberg et al., 1994). Tungstate and molybdate, however, facilitate bioeffects in rat adipocytes only at high (millimolar) concentrations (Goto et al., 1992). We report here that incubations of tungstate or molybdate with hydrogen peroxide (H_2O_2) result in the formation of pertungstate (pW, peroxide of tungstate) or permolybdate (pMo, peroxide of molybdate). Pertungstate and permolybdate were found to stimulate all or most of the insulin bioeffects in rat adipocytes. Moreover, these permetallooxides are 80−180-fold more potent stimulators than the corresponding metallooxides. This shift in potency resembles that of pervanadate relative to vanadate in stimulating the same effect in rat adipocytes (Fantus et al., 1989). pW and pMo are also active in normalizing blood glucose levels in streptozotocin-induced diabetic rats. Further studies aimed at understanding the higher efficacy of this permetallooxide revealed the following: (a) All three permetallooxides (pV, pW, pMo) are oxidizing agents relative to reduced glutathione (GSH). They oxidize stoichiometric amounts of GSH to GSSG. (b) All three metallooxides do not oxidize GSH to GSSG. (c) Both metallooxides and permetallooxides inhibit rat adipocytic PTPase at micromolar quantities ($IC_{50} = 3-10\ \mu M$). Permetallooxides, however, inhibited a larger PTPase fraction (80−100%) compared to metallooxides (40−70% of the total). (d) In cells, metallooxides solely activate cytPTK, whereas permetallooxides activate both cytPTK and the insulin receptor tyrosine kinase (insRTK). (e) Although both protein tyrosine kinases are stimulated, permetallooxides mediate their effects predominantly via the insulin receptor. (f) GSH partially protects PTPase from metallooxide inhibition in cell-free experiments. We propose that the higher efficacy of permetallooxides in stimulating insulin responses originates from their oxidizing feature relative to GSH. Partial intracellular conversion of GSH to GSSG resulted in the inhibition of higher PTPase fraction, activation of the insRTK, and efficient triggering of insulin bioeffects via the activated receptor in a hormone-independent manner.

It was suggested that the insulin-like actions of vanadate are not mediated via the insulin receptor tyrosine kinase (Fantus et al., 1989; Strout et al., 1989; Mooney et al., 1989; Shechter et al., 1989; Venkatesan et al., 1991). On the other hand, reports indicate that endogenous tyrosine phosphorylation of cellular protein substrates is an early and essential event in facilitating hexose uptake and glucose metabolism by insulin and vanadate (Rosen, 1987; Shechter et al., 1989; Shoelson & Kahn, 1989; Shechter, 1990; Shisheva & Shechter, 1992; White & Kahn, 1994). We have identified a 53 kDa cytosolic protein tyrosine kinase (cytPTK)[1] in rat adipocytes. The enzyme was stimulated in vanadate-pretreated adipocytes, and both cytPTK activity and vanadate stimulation of glucose metabolism in this cell type were inhibited by staurosporine (Shisheva & Shechter, 1991, 1993). Staurosporine did not quench the effects of vanadate in stimulating glucose uptake or in inhibiting lipolysis. This suggested that the former bioeffect was not mediated via cytPTK and antilipolysis is an insulin (and vanadate) event that may not require intracellular tyrosine phosphorylation at all (Shechter et al., 1989; Shisheva & Shechter, 1992). Further studies have revealed that activation of cytPTK by vanadate is also preserved after cell disintegration. Vanadate activated cytPTK in the 40 000*g* supernatant fraction of rat adipocytes with ED_{50} value = 3.0 ± 0.7 μM, and this activation could also be produced by tungstate and molybdate

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[1] Abbreviations: cytPTK, cytosolic protein tyrosine kinase; insRTK, insulin receptor tyrosine kinase; BSA, bovine serum albumin; Poly(Glu$_4$Tyr), poly[(GluNa:Tyr)4:1]; KRB, Krebs−Ringer bicarbonate; STZ, streptozotocin; STZ-rats, rats made diabetic with streptozotocin; pNPP, *p*-nitrophenyl phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); V, vanadate; W, tungstate; Mo, molybdate; pV, pervanadate; pW, pertungstate; pMo, permolybdate; GSSG, oxidized glutathione; PTK, protein tyrosine kinase.

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(Elberg et al., 1994). We have further demonstrated that the common denominator for all three metallooxides is their ability to inhibit rat adipocytic PTPase, when the substrate utilized is *p*-nitrophenyl phosphate. As expected from these cell-free experiments, tungstate and molybdate mimicked the biological actions of insulin *in vitro* and *in vivo* via the vanadate-dependent pathways.[2] A major difference, however, between vanadate and the other two metallooxides was that, in intact adipocytes, the insulin effects were produced only at high (millimolar) concentrations of tungstate and molybdate.

It has previously been demonstrated that vanadate interacts with H_2O_2 to form pervanadate (peroxide of vanadate). The structure and basic features of pervanadate have not yet been fully elucidated. However, it was found to be about 100-fold more potent than vanadate in stimulating the biological actions of insulin in rat adipocytes (Fantus et al., 1989). Goto et al. (1992) observed a synergistic effect of tungstate and molybdate in activating glucose uptake in the presence of H_2O_2. Our aims, in this study, were to determine whether interaction of tungstate and molybdate with H_2O_2 will form the respective permetallooxides and, if positive, to ascertain their basic features, their potency as insulin mimickers, and their modes of action.

MATERIALS AND METHODS

Materials. D-[U-^{14}C], D-[1-^{14}C], and D-[6-^{14}C]glucose and D-3-*O*-[*methyl*-^{14}C]glucose were purchased from New England Nuclear (Boston, MA). Collagenase type I (134 U/mg) was obtained from Worthington Biochemicals (Freehold, NJ). Porcine insulin was purchased from Eli Lilly Co. (Indianapolis, IN). Phloretin, 3-*O*-methyl-D-glucose, D,L-isoproterenol hydrochloride, quercetin, poly[(GluNa:Tyr)4:1] [poly(Glu$_4$Tyr)], reduced glutathione (GSH), and bovine liver catalase (thymol-free) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium molybdate ($Na_2MoO_4{\cdot}2H_2O$, MW =241.95) was from BDH Chemicals Ltd., and sodium tungstate ($Na_3WO_4{\cdot}2H_2O$, MW = 329.9) was purchased from General Chemical Division-Allied Chemical and Dye Corp., NY. Aqueous solutions of 100 mM (24.2 mg/mL sodium molybdate, 33 mg/mL sodium tungstate) were prepared and adjusted with HCl to pH 7.0 and kept at 7 °C until used. Affinity-purified monoclonal antibodies to phosphotyrosine were kindly donated by Mono-Yeda (Rehovot, Israel). Krebs−Ringer bicarbonate (KRB) buffer, pH 7.4, contained NaCl, 110 mM; $NaHCO_3$, 25 mM; KCl, 5 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 1.3 mM; and $MgSO_4$, 1.3 mM.

Streptozotocin-Treated Rats. Diabetes was induced by a single intravenous injection of a freshly prepared solution of streptozotocin (55 mg/kg of body weight) in 0.1 M citrate buffer (pH 4.5). The effect of pW or pM on blood glucose levels was determined 3 months following induction of diabetes.

Cell Preparation and Bioassays. Rat adipocytes were prepared from fat pads of male Wistar rats (100−200 g) by collagenase digestion (Rodbell, 1964). Cell preparations showed more than 95% viability by trypan blue exclusion, at least 3 h after digestion. All bioassays were performed as described in the figure legends. Glucose transport was carried out using 3-*O*-[*methyl*-^{14}C]glucose (Whitesell & Gliemann, 1979). Glucose oxidation was measured by conversion of D-[U-^{14}C], D-[1-^{14}C], or D-[6-^{14}C]glucose to $^{14}CO_2$ (Rodbell, 1964) and *lipogenesis* by incorporation of the ^{14}C-labeled glucose analogs into lipids according to Moody et al. (1974). Lipolysis and antilipolysis were evaluated by measuring the glycerol released, as described previously (Shechter, 1982).

High-speed supernatant fractions, as a source for cytPTK and PTPase activity, were prepared as follows. Fresh adipocytes were washed three times with KRB buffer, pH 7.4, containing 0.7% BSA and then two times more with 50 mM HEPES buffer, pH 7.4, containing 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 5 μg/mL aprotinin. The cells (about 10 mL of packed adipocytes) were homogenized with a manual Teflon homogenizer and then frozen and thawed five times. The fat was removed and the cell homogenate centrifuged at 40000*g* (Centrikon T-1065, Kontron Instruments) for 60 min. The supernatant was divided into aliquots and stored at −137 °C. This crude cell extract was used as a source for cytPTK activity.

Tyrosine Kinase Activity Measurements. The assay mixture (final volume of 60 μL in 50 mM HEPES buffer, pH 7.4) contained 0.5−5 μg of cytosolic adipocytic protein, 20 mM $MgSO_4$, 1 mM $CoCl_2$, and 100 μM ATP. Following a 20 min preincubation at 22 °C, the reaction was initiated by adding poly(Glu$_4$Tyr) (15 μg/assay mixture). This was proceeded for 30 min and terminated by adding EDTA (30 mM). The effect of metallooxides and permetallooxides on cytPTK activity in intact cells was evaluated by preincubating freshly prepared adipocytes (~1 × 10^6 cells) in 0.5 mL KRB buffer −0.7% BSA (pH 7.4), for 30 min at 37 °C with the indicated concentrations of metallooxides or permetallooxides. The cells were then frozen and thawed four times. Fat was removed and the cell homogenate centrifuged at 40000*g* for 30 min. Aliquots from the supernatant fraction (1−20 μL) were then assayed. Phosphotyrosine content in poly(Glu$_4$Tyr) was quantitated by a radioimmunoassay procedure (Shisheva et al., 1991). This assay included specific monoclonal antibodies to phosphotyrosine (final dilution of 1:100.000) and [^{125}I]BSA−phosphotyrosine conjugate.

PTPase activity measurement was estimated by the potency of rat adipocytic cytosol (20 μg protein/mL) to hydrolyze *p*-nitrophenyl phosphate (pNPP). The assay (0.2 mL) consisted of pNPP (5 mM in 0.05M Tris-HCl, pH 7.5), the enzyme source, and the indicated concentrations of metallooxides or permetallooxides. The extent of hydrolysis was determined by spectroscopy at 405 nm, as described by Kremerskothen and Barnekow (1993).

Protein concentration was determined by the Bradford method (Bradford, 1976).

Oxidizing Capacity of Permetallooxides. An aliquot (9 μL) from permetallooxide solution (10 mM) was added to 1 mM GSH (0.1 mL) in 50 mM Hepes buffer, pH 7.4. Following a 10 min incubation, the solution was diluted 10-fold with 50 mM Hepes (pH 7.4), and the GSH remaining was quantitated with DTNB, according to Ellman (1959), using molar extinction, ϵ_{412} = 13 600. All the assays were performed in duplicate or triplicate. The data in figures and tables are presented as the means ± SE from at least three separate experiments.

[2] J. Li, G. Elberg, J. Libman, A. Shanzer, D. Gefel, and Y. Shechter, submitted.

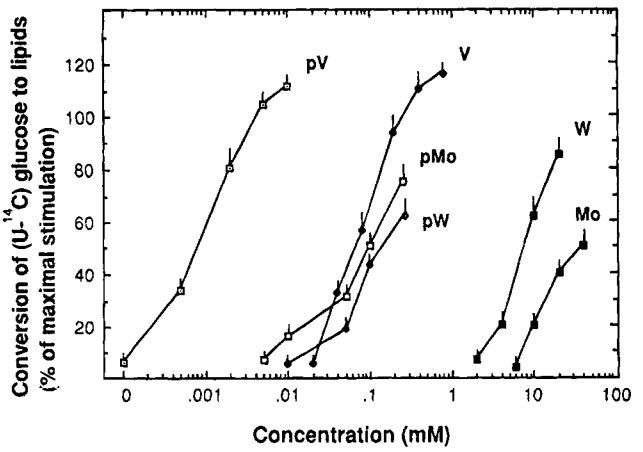

FIGURE 1: Concentration-dependent stimulation of lipogenesis by metallooxides and permetallooxides. Freshly prepared adipocytes (2×10^4 cells/mL suspended in KRB buffer, pH 7.4, containing 0.7% BSA) were preincubated for 20 min with the indicated concentrations of the metallooxides or permetallooxides. The cells were then supplemented with [U-^{14}C]glucose (final concentration of 0.16 mM), and lipogenesis was performed for an additional 1 h at 37 °C. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

RESULTS

Permetallooxides of Tungstate and Molybdate Are Potent Stimulators of Lipogenesis in Rat Adipocytes. In preliminary experiments, all three metallooxides (in 50 mM Hepes buffer, pH 7.4) were incubated with 5 M excess H_2O_2 for 10 min at 24 °C. Catalase was then added (final concentration of 20 μg/mL) just shortly before the lipogenesis assay. Catalase was previously shown to destroy excess H_2O_2 solely, leaving the pervanadate formed unaltered (Fantus et al., 1989). Figure 1 shows stimulation of lipogenesis at increasing concentrations of all six compounds. As can be seen in the figure, the products of tungstate or molybdate with H_2O_2 exhibited dramatic left-shifted dose–response curves. Thus, ED_{50} values (calculated from Figure 1) were 0.08 ± 0.01, 7.0 ± 0.4, and 11 ± 0.3 mM for V, W, and Mo and 1 ± 0.2, 60 ± 5, and 70 ± 4 μM for pV, pMo, and pW, respectively, indicating that permetallooxides are 80–180-fold more potent stimulators of lipogenesis as compared to the parent metallooxides. It should be noted, however, that the extent of stimulation by W and Mo or pW and pMo, as compared to insulin, is lower (50–80% of maximal). Unlike V, Mo, and W, preincubating the cells with permetallooxides for 20 min at 37 °C prior to the assay neither altered ED_{50} values nor increased the extent of stimulation of lipogenesis (not shown).

Basic Difference between Metallooxides and Permetallooxides. Figure 2 demonstrates that permetallooxides are oxidizing agents relative to reduced glutathione. Following preparation of pV, pW, and pMo (5 M excess of H_2O_2 and catalase), aliquots were added to GSH solutions. The addition of 0.5 or 1 mol of each permetallooxide prepared consumed stoichiometric quantities of GSH (Figure 2). In contrast, V, Mo, or W at 3 M excess over glutathione (or even at 10 M excess, not shown) did not consume GSH at all. Thus, permetallooxides differ from metallooxides in being stoichiometric oxidizing agents of GSH.

We have further determined the formation of permetallooxides as a function of H_2O_2 added and the stability of the products in aqueous solutions under different experimental conditions. Figure 3 shows the extent of formation of the three permetallooxides at pH 7.4 as a function of the quantity of H_2O_2 added. Conversion of V to pV was complete upon adding 1.2 mol of H_2O_2. W and Mo required 2.4 ± 0.1 and 3.0 ± 0.15 mol of H_2O_2, respectively, to bring the reaction to completion. Thus, the extent of permetallooxide formation

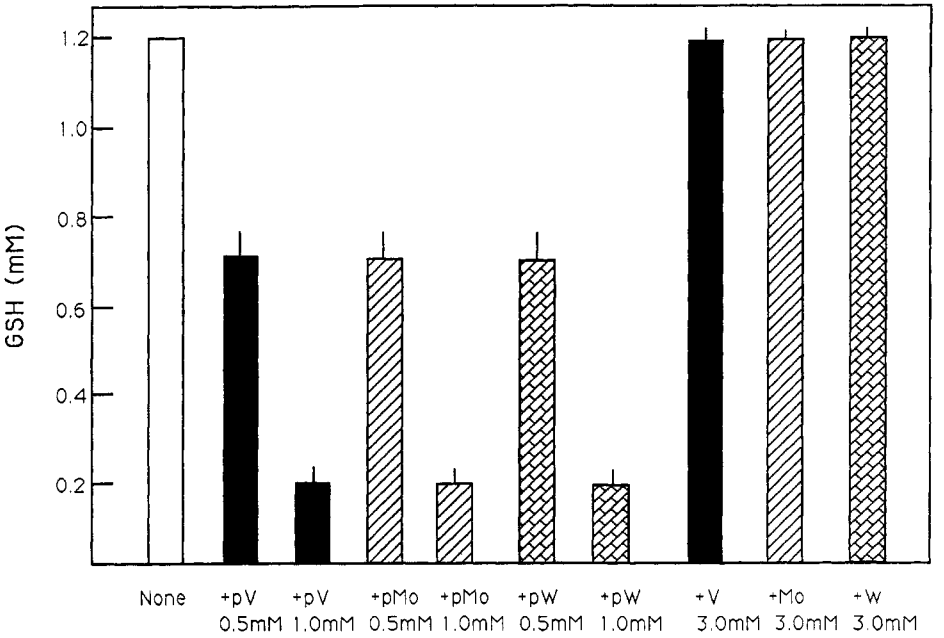

FIGURE 2: Permetallooxides, but not metallooxides, consumed stoichiometric quantities of reduced glutathione. Permetallooxides were prepared by incubating aqueous solutions of vanadate, tungstate or molybdate (10 mM) with 5 M excess of H_2O_2. Following addition of H_2O_2, solutions were adjusted to pH 6 with diluted HCl. Excess H_2O_2 was destroyed by catalase (20 μg/mL, 5 min) and aliquots (5 or 10 μL of permetallooxides or 30 μL of metallooxides) were added to solutions of GSH (100 μL of 1.1 mM GSH in 50 mM Hepes buffer, pH 7.4). Following a 20 min incubation, the solution was diluted 10-fold with 50 mM Hepes buffer, pH 7.4, and DTNB (10 μL from 0.1 M solution in DMF) was added. The absorbance at 412 nm was recorded. The amount of GSH in solution was determined according to Ellman (1959), using a molar extinction of $\epsilon_{412} = 13\,600$. Controls containing H_2O_2 and catalase did not consume glutathione.

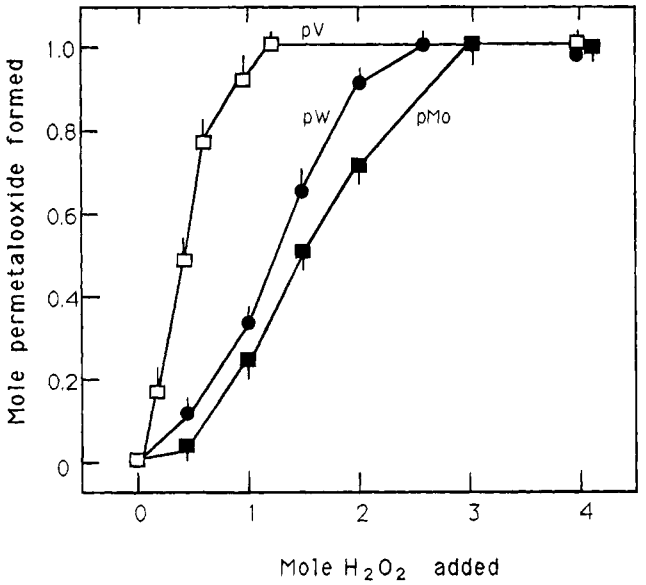

FIGURE 3: Formation of pervanadate, pertungstate, and permolybdate as a function of increasing concentration of H_2O_2. Increasing concentrations of H_2O_2 were added to aqueous solutions of vanadate, tungstate, or molybdate (10 mM, pH 6.0). Following a 10 min incubation, catalase was added (20 μg/mL) and aliquots (9 μL) were withdrawn to determine the amount of GSH consumed, as specified in the legend to Figure 2. Results are expressed as mol of permetallooxides formed/mol of H_2O_2 added. The amount of permetallooxide formed was calculated based on the 1:1 molar stoichiometry of GSH oxidized/permetallooxides formed.

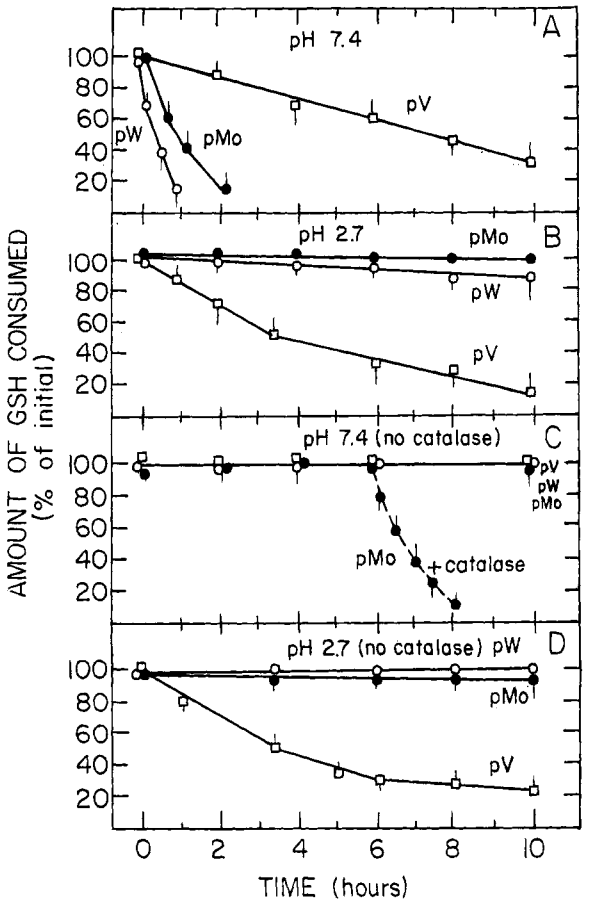

FIGURE 4: Stability of permetallooxides at different pH values, effect of catalase. Permetallooxides were basically prepared by adding 5 M excess of H_2O_2 to a 10 mM aqueous solution (H_2O, pH 6.0) of either V, Mo, or W. Catalase was then added (20 μg/mL, 5 min). (A) Permetallooxides were then brought to pH 7.4, and aliquots (9 μL) were taken at the indicated time points for determining GSH-oxidizing capacity (as described in detail in the legends to Figures 2 and 3). (B) Solutions of permetallooxides were reduced to pH 2.7 (with acetic acid), and aliquots were withdrawn to determine GSH-oxidizing capacity as above. (C) Following addition of catalase, permetallooxides were dialyzed against 9 volumes of H_2O for 4 h at 15 °C. The dialyzed external solution (containing permetallooxides but no detectable catalase) was brought to pH 7.4 or to 0.1 M acetic acid (in D), and aliquots (90 μL) were taken for determining GSH-oxidizing capacity as above. Dashed line (panel C) represents the addition of catalase (20 μg/mL) to permolybdate followed by determination of its GSH-oxidizing capacity over a period of 2 h. 100% was taken at time 0.

following addition of H_2O_2 was in the order of pV > pW ≥ pMo.

With respect to the permetallooxide stability in aqueous solutions at different pH values, this was initially studied at pH 7.4. Following the addition of catalase, aliquots were taken at different time intervals for determining the capacity of permetallooxides to oxidize GSH. Under these conditions, pV decayed with $t_{1/2}$ value = 7.0 ± 0.5 h, whereas pW and pMo exhibited even shorter half-lives ($t_{1/2}$ = 1.0 ± 0.2 and 2 ± 0.3 h, respectively; Figure 4A). pW and pMo were found to be fully stable at a lower pH (i.e., 2.7). In contrast, pV exhibited lower stability at acidic pH values ($t_{1/2}$ = 3.5 ± 0.3 h; Figure 4B). At this stage, we suspected that permetallooxides are sensitive to catalase to a certain extent. Therefore, the enzyme was removed several minutes after its addition (experimental details in legend to Figure 4). Following removal of catalase, all three permetallooxides were found to be highly stable at pH 7.4 (Figure 4C), as well as at higher pH values (not shown). We further tested the stability of permetallooxides in the absence of catalase at pH 2.7 and found pW and pMo to be highly stable and pV to decay with $t_{1/2}$ = 3.3 ± 0.3 h (Figure 4D). The effect of catalase in decomposing pW at pH 7.4 was reconfirmed by adding the enzyme (20 μg/mL). In the presence of the enzyme, the GSH-oxidizing capacity of pMo decayed within 2 h (dashed line, Figure 4C). In summary, pW and pMo were found to be stable in aqueous solution at any pH tested in the absence of catalase. pV is highly stable at neutral and alkaline pH values but slowly decayed at acidic pH.

Effect on PTPase Activity. All three permetallooxides inhibited rat adipocytic PTPase activity in hydrolyzing pNPP in cell-free experiments. IC_{50} values amounted to 3.3 ± 0.2, 6 ± 0.4, and 10 ± 0.7 μM for pV, pW, and pMo, respectively (Figure 5). These values do not differ drastically from those obtained previously for V, W, and Mo, but a larger PTPase fraction is inhibited by the permetallooxide as compared to metallooxides (not shown). In the presence of a nucleophile such as hydroxylamine, PTPase activity is increased by ~2-fold. Hydroxylamine reverses PTPase inhibition by vanadate and vanadyl (not shown) but has no effect at all on the inhibitory potency of Mo or W (Table 2).

Reduced Glutathione Protects PTPase from Vanadate Inhibition. The results in Figure 6 show that GSH significantly protects PTPase activity from being inhibited by vanadate. The IC_{50} value was shifted from 1.1 ± 0.1 to 4.9 ± 0.2 μM for vanadate inhibition, in the absence and presence of 1 mM GSH, respectively (Figure 6). Also, the total PTPase-inhibited fraction, that was 74% in the absence of GSH, reduced to 53% in its presence. Thus, the presence of GSH in the assay seems to significantly protect PTPase from being inhibited by vanadate.

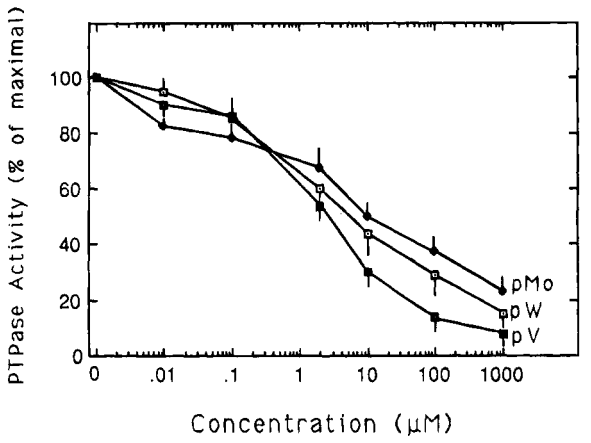

FIGURE 5: Inhibition of rat adipocytic protein phosphotyrosine phosphatase activity by permetallooxides. The reaction was carried out for 30 min at 37 °C. The assay included (in 0.2 mL of 0.1 M Tris-HCl, pH 7.5) pNPP (5 mM), the indicated concentrations of pervanadate, pertungstate, or permolybdate, and an aliquot (20 μg of protein) from the 40000*g* supernatant fraction derived from rat adipocytes as the enzyme source. This was preincubated for several minutes with the metallooxides prior to the addition of pNPP.

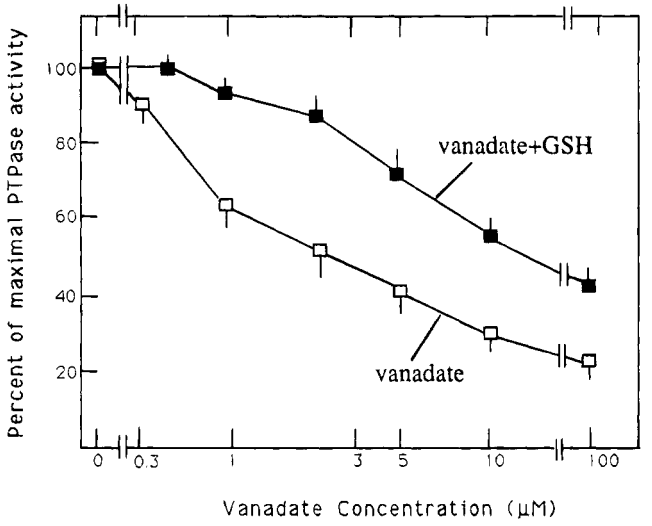

FIGURE 6: Inhibition of rat adipocytic PTPase by vanadate in the presence and absence of GSH. Hydrolysis of pNPP by rat adipocytic cytosol (20 μg/protein) was performed in the presence of the indicated concentrations of vanadate in the absence (□) or presence (■) of 1 mM GSH. The reaction was carried out for 30 min at 37 °C, as described in the legend to Figure 5. GSH alone (1 mM) had no effect on PTPase activity.

pW and pMo Are Stimulators of Insulin Effects in Rat Adipocytes. In addition to stimulating lipogenesis (Figure 1), pW and pMo were also found to stimulate hexose uptake and glucose oxidation both via glycolysis and hexose monophosphate pathways and to inhibit isoproterenol-mediated lipolysis (Table 1). A synergistic effect on hexose uptake was evident when the cells were treated with insulin and pW. It is important to note that the effect of insulin in inhibiting lipolysis occurs via a mechanism that is different from the hormonal effects relating to activate hexose uptake or glucose metabolism [i.e., Shechter (1984); Amir et al. 1987; Shisheva & Shechter 1993a]. Nevertheless, pV, pMo, and pW mimic both these insulin-like effects. Somewhat higher concentrations, however (0.05 ± 0.01, 0.4 ± 0.04, and 0.55 ± 0.05 mM, respectively), were required to inhibit lipolysis by pV, pMo, and pW, as opposed to the lower concentrations at which these permetallooxides stimulated lipogenesis (Figure 1).

Table 1: Insulin-like Effects of Permetallooxides—Stimulation of Hexose Uptake, Glucose Oxidation and Inhibition of Lipolysis (Mean ± SEM, $n = 4$)

Hexose Uptake[a]

	3-*O*-methyl-glucose uptake (fmol/min/cell)	insulin response (%)
basal	0.38 ± 0.02	0
insulin (17 nM)	1.81 ± 0.1	100
pertungstate (250 μM)	0.81 ± 0.04	30
pertungstate (250 μM) plus insulin (17 nM)	2.36 ± 0.15	138
permolybdate (250 μM)	1.53 ± 0.07	64
permolybdate plus insulin (17 nM)	1.73 ± 0.1	95

Glucose Oxidation[b]

	[1-^{14}C]glucose oxidation to $^{14}CO_2$ (nmol/h/10^4 cells)	insulin response (%)
Via Pentose Phosphate Pathway		
basal	1.5 ± 0.08	0
insulin (17 nM)	5.55 ± 0.2	100
pertungstate (250 μM)	4.35 ± 0.15	70
permolybdate (250 μM)	3.53 ± 0.14	50
Via Glycolysis	[6-^{14}C]glucose oxidation to $^{14}CO_2$ (nmol/h/10^4 cells)	
basal	0.8 ± 0.03	0
insulin (17 nM)	4.8 ± 0.2	100
pertungstate (250 μM)	1.68 ± 0.09	22
permolybdate (250 μM)	2.64 ± 0.2	46

Inhibition of Isoproterenol Mediated Lipolysis

		glycerol-released (nmol/3 × 10^5 cells/h)	inhibition of lipolysis (%)
none		8 ± 0.2	
isoproterenol (30 μM)		122 ± 5	
isoproterenol + pV	(10 μM)	100 ± 6	20
	(50 μM)	63 ± 2	52
	(200 μM)	48 ± 2	65
	(500 μM)	30 ± 1.3	81
isoproterenol + pW	(10 μM)	110 ± 6	11
	(200 μM)	71 ± 2	45
	(2000 μM)	30 ± 1.5	81
isoproterenol + pMo	(200 μM)	91 ± 4	27
	(700 μM)	60 ± 2	54
	(2000 μM)	42 ± 3	70

[a] According to Whitesell and Gliemann (1979). Suspensions of adipocytes (30%) in KRB buffer (pH 7.4)−1% BSA were preincubated for 20 min at 37 °C with the indicated concentrations of insulin or permetallooxides prior to transferring aliquots to tubes containing 3-*O*-[methyl-^{14}C]glucose (50 000 cpm/nmol, final concentration 0.1 mM). [b] According to Rodbell (1964). Adipocytes were preincubated with the indicated concentrations of insulin or permetallooxides for 20 min at 37 °C and then supplemented with either [6-^{14}C]- or [1-^{14}C]glucose (final concentration 0.15 mM). Assay was performed for 1 h at 37 °C.

pMo and pW Normalize Blood Glucose Levels in STZ-Treated Diabetic Rats. A single ip injection of pW or pMo (0.1 mol/kg each) reduced blood glucose levels in STZ-treated hyperglycemic rats. The effect persisted over a period of 2−8 h following administration (Figure 7). Within this time frame (2−8 h), the blood glucose level of control STZ-rats deprived of food was not significantly decreased (Figure 7). In a previous study, W was found to be effective as well in inducing a similar effect (not shown). A rough

Table 2: Inhibition of Rat Adipocytic PTPase by Vanadate, Molybdate, and Tungstate in the Presence and Absence of Hydroxylamine

	mmol of pNPP hydrolyzed/mg of protein/h at 37 °C[a]	initial activity (%)
no enzyme added	0.01 ± 0.001	0
PTPase	0.13 ± 0.01	100
PTPase + vanadate (10 μM)	0.048 ± 0.002	39
PTPase + tungstate (30 μM)	0.052 ± 0.004	42
PTPase + molybdate (30 μM)	0.055 ± 0.05	45
PTPase + NH_2OH	0.28 ± 0.03	100
PTPase + NH_2OH + vanadate (10 μM)	0.27 ± 0.01	96
PTPase + NH_2OH + tungstate (30 μM)	0.13 ± 0.01	44
PTPase + NH_2OH + molybdate (30 μM)	0.12 ± 0.01	41

[a] pNPP hydrolysis was carried out for 1 h at 37 °C in 0.1 mL of 50 mM Tris-HCl buffer (pH 7.5). The assay contained pNPP (5 mM) and the enzyme source (rat adipose—40 000*g* superantant, 20 μg/mL) and was performed in the presence and absence of 50 mM hydoxylamine (NH_2OH).

calculation seems to indicate that pW is ~10-fold more active than W in reducing blood glucose levels *in vivo*.

Mechanism of pMo- and pW-Dependent Stimulation in Rat Adipocytes. To gain insight into the mechanism(s) by which pMo, pV, and pW facilitate the actions of insulin in the intact cell system, we first analyzed these compounds to see whether they activate cytPTK, as does vanadate (Shisheva & Shechter, 1991, 1993). In this set of experiments, fresh intact adipocytes were preincubated with the tested compounds. The cells were then lysed, and the specific activity of cytPTK in the 40000*g* supernatant was determined (Shisheva & Shechter, 1991, 1993). Similarly to vanadate, pV, pW, and pMo also stimulated the enzyme to either a larger or smaller extent (Figure 8). Using quercetin (Shisheva & Shechter, 1992) or staurosporine (Shisheva & Shechter, 1991, 1993), we could differentiate whether a given agent or condition facilitates its insulin-like effects via the insRTK pathway (inhibited by quercetin) or, alternatively, through the cytPTK (inhibited by staurosporine). Figure 9 shows activation of lipogenesis, triggered by either insulin, V, pMo, or pW. Quercetin inhibited lipogenesis induced by insulin and the three permetallooxides tested but not that induced by vanadate. In contrast, insulin and the permetallooxides studied were only slightly inhibited by staurosporine. Thus, in intact cells, the stimulating effects of the permetallooxides seem to proceed predominantly via the insulin receptor pathway. This is in spite of the fact that cytPTK is also activated in permetallooxide-pretreated adipocytes (Figure 8).

DISCUSSION

There is growing basic and clinical interest in agents mimicking the biological actions of insulin. This has been validated for those producing insulin effects via an alternative (insulin-independent) pathway, as suggested for vanadate (Shisheva & Shechter, 1991, 1993) and, more recently, for tungstate and molybdate. Not less interesting are agents facilitating insulin effects via the insulin receptor itself but in a hormone-independent intracellular mode. Such a route is suggested here for pV, pW, and pMo. These two classes of insulin mimickers may be beneficial in overcoming states of insulin resistance, a syndrome likely to originate from several defects along the insulin-dependent pathways, both at the receptor and postreceptor levels (Kahn et al., 1977; Marshall & Olefsky, 1980; Kosmakos & Roth, 1980; Olefsky & Kolterman, 1981; Garvey, 1989).

In this study, we have introduced two new insulin mimickers, namely pW and pMo, that were found to form upon interaction of W and Mo with H_2O_2 (Figures 2, 3). pW and pMo appeared to be highly stable in aqueous solution at various pH values (Figure 4B–D), to produce virtually most or all insulin effects in rat adipocytes (Figures 1, Table 1), and also to be active in inducing normoglycemia in STZ-rats (Figure 7). The observation of the 80–180-fold higher

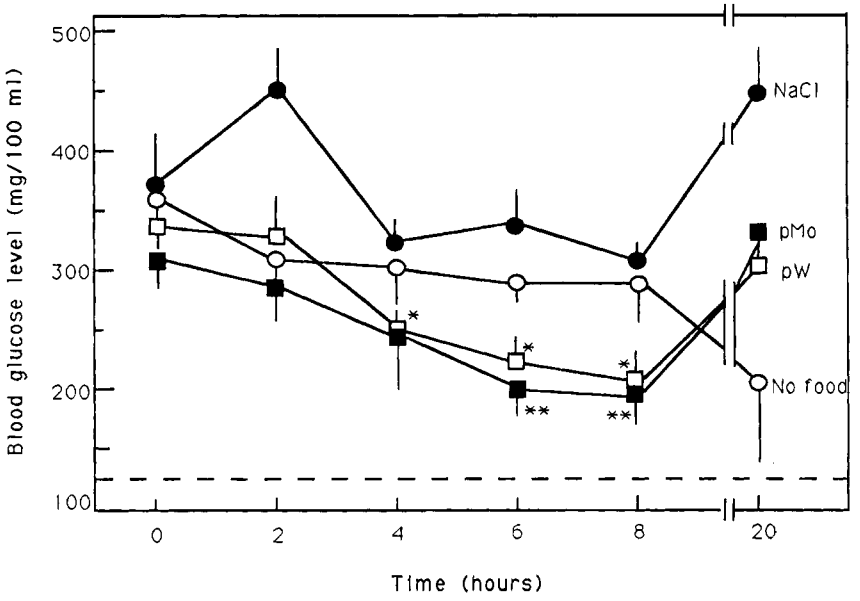

FIGURE 7: Effect of pertungstate and permolybdate administration on blood glucose levels of streptozotocin-treated hyperglycemic rats. Hyperglycemic STZ-rats, 3 months after induction of diabetes, were injected intraperitoneally with NaCl (●), pW (□) or pMo (■) (0.1 mol/kg each). Blood glucose levels were determined over 0–20 h following administration. A group of rats deprived of food was also included (○). Each group consisted of four rats ($n = 4$). Each point represents the arithmetic mean ± SEM of plasma glucose for four rats. The student's *t*-test was applied to compare control groups (NaCl-treated or food-deprived) to pW- and pMo-treated groups. $P < 0.05$ (*) or $P < 0.01$ (**) compared with food-deprived or NaCl-treated group. The horizontal dashed line indicates the arithmetic mean for plasma glucose in control (nondiabetic) rats.

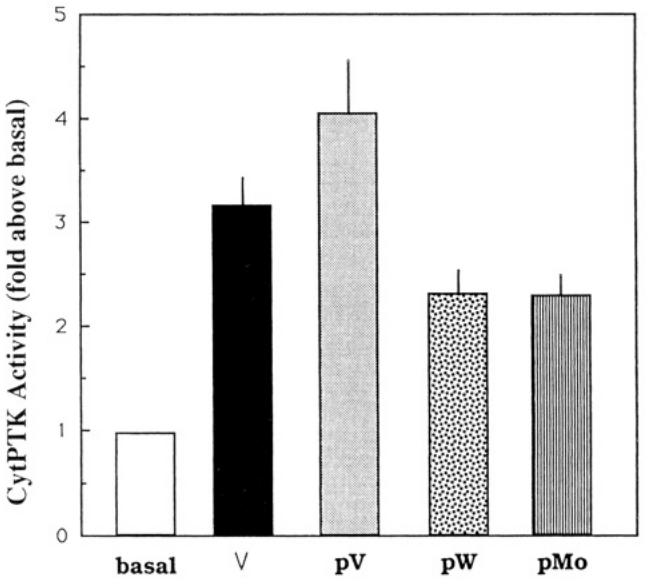

FIGURE 8: Stimulation of cytPTK activity by permetallooxides in intact adipocytes. Freshly prepared rat adipocytes were preincubated in the presence or absence of either vanadate (1 mM), pV (20 μM), pMo (0.25 mM), or pW (0.25 mM) for 20 min at 37 °C. The cells were then lysed, and the specific activity of cytPTK in the 40 000g supernatant fraction was determined.

insulinomimetic efficacy in cells of pMo and pW, in comparison to Mo and W (Figure 1), led to further studies which appeared to indicate that permetallooxides differ from metallooxides in being stoichiometric oxidizing agents of GSH (Figure 2) and in their potency to significantly inhibit a greater rat adipocytic PTPase fraction in cell-free experiments (Figure 5).

The above experimental findings raised four new basic questions, of which a major part was tackled here, in greater detail, and the rest is the basis of our future working hypothesis. For the sake of discussion, the four questions are given here in a rational order: (1) What is the origin of the 100-fold higher insulinomimetic potency of permetallooxides relative to the metallooxides? (2) Why do metallooxides activate only cytPTK? (3) Why do permetallooxides activate both the receptor and the cytosolic enzyme? (4) If both enzymes are activated, why do permetallooxides facilitate their bioeffects in cells predominantly via the receptor pathway? With respect to issue 1, the 100-fold higher potency is related, with a high degree of confidence, to the oxidizing feature of the permetallooxides relative to metallooxides (Figures 2, 3), a fact appearing to be directly linked to the higher degree of PTPase inhibition. This more extensive inhibition was validated both in a cell-free system (Figure 5) and even more so in cells in which vanadate exhibited negligible inhibition and pervanadate (at 10−100 μM) inhibited 60−90% of the total PTPase activity [Figure 7A in Shisheva and Shechter (1993b)]. It should be noted at this point that rat adipocytic PTPase is an SH-containing protein. Moreover, the free sulfhydryl group(s) appear to participate in catalysis (Guan & Dixon, 1991), as concluded by the potency of chloramine T to reduce rat adipocytic PTPase activity (manuscript in preparation). Vanadate renders this group susceptible to oxidation to sulfenic acid (manuscript in preparation). That GSH reduces the potency of vanadate to inhibit PTPase (Figure 6) may suggest that the cysteinyl group of the enzyme participating in catalysis is more susceptible to oxidation. The mammalian cell contains endogenous GSH in millimolar quantities to maintain the cellular redox state and is likely to contribute in preserving the endogenous PTPase in its reduced and active form. Our observations relating to the GSH-oxidizing capacity of the permetallooxides (Figures 2, 3) suggest the occurrence of such GSH oxidation in permetallooxide-pretreated cells as well, rendering PTPase more susceptible to permetallooxide inhibition. How micromolar concentrations of permetallooxides oxidize millimolar quantities of intracellular GSH is of no surprise. Under the experimental system we are using, namely ~5% fat cell suspension of

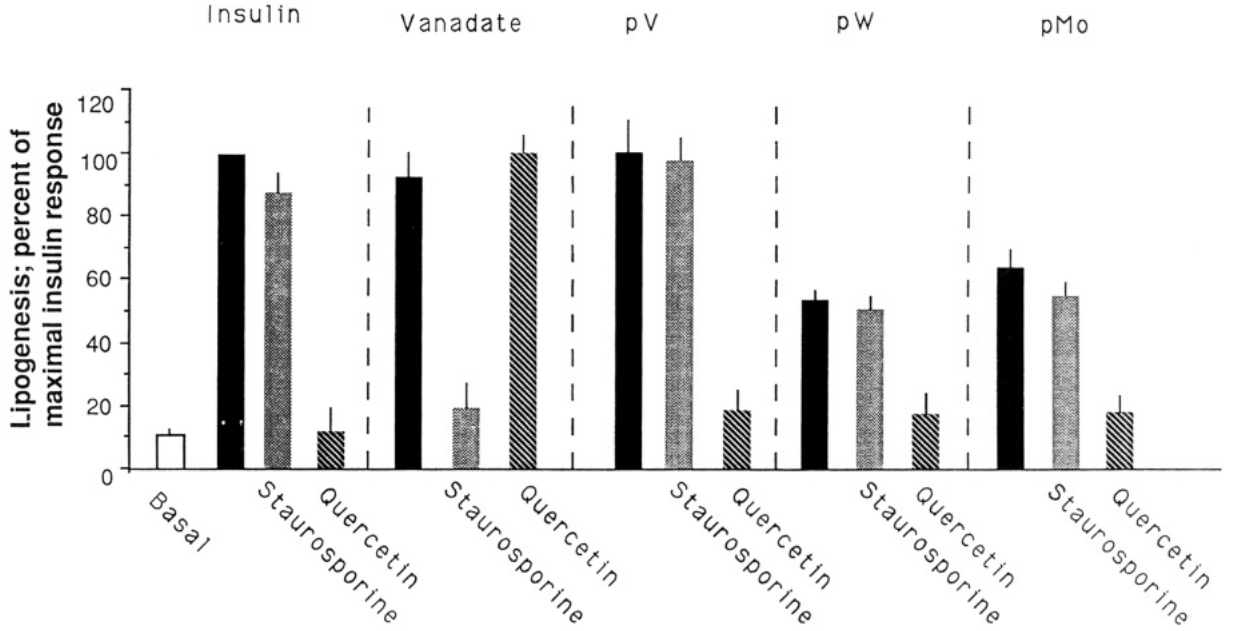

FIGURE 9: Effects of staurosporine and quercetin on permetallooxide-mediated lipogenesis. Adipocytes were preincubated for 20 min at 37 °C in the presence or absence of either quercetin (150 μM) or staurosporine (0.3 μM). Lipogenesis was then carried out for 1 h at 37 °C with [U-^{14}C]glucose (final concentration of 0.16 mM) in the presence or absence of either insulin (17 nM), vanadate (1 mM), pertungstate (0.25 mM), or permolybdate (0.25 mM).

small cytoplasmic volume (Czech, 1977), the GSH concentration in the suspension is in the micromolar range as well.

With respect to questions 2 and 3, our studies support the contention that both protein tyrosine kinases (PTKs) are under the regulatory influence of the cellular PTPase (Posner et al., 1994), which eliminates spontaneous self-phosphorylation and activation of the kinases. In the case of the insulin receptor, PTPase is likely to play an essential role in terminating receptor activation after dissociation of insulin [i.e. Meyerovitch et al. (1992)]. Partial inhibition activates cytPTK, whereas more extended inhibition activates both PTKs. We have observed that the potency of vanadate to inhibit PTPase is largely dependent on the substrate to be dephosphorylated (manuscript in preparation). This can explain why vanadate activates only cytPTK. We suggest here that metallooxides effectively block PTPase when the substrate is phosphorylated cytPTK, leaving insRTK dephosphorylation undisturbed. When PTPase is inhibited to a larger extent (i.e., by permetallooxides), insRTK dephosphorylation becomes less efficient, resulting in activation of the insRTK as well. Although a significant decrease in PTPase activity is sufficient to explain the receptor activation, the possibility that the insRTK also becomes a weaker substrate for the PTPase due to permetallooxide-dependent alterations (such as GSH oxidation) cannot be excluded.

It should be noticed that in intact adipocytes vanadate is about 2 orders of magnitude more potent than W and Mo as an insulinomimetic agent and the same ratio is preserved when one compares pV to pW or pMo (Figure 1). This is in spite of the fact that all three permetallooxides have the same GSH-oxidizing potency (Figure 2) and all six compounds do not exhibit such differences in inhibiting PTPases in cell-free experiments (Figure 5 and not shown). We still do not know the answer for this discrepancy. Perhaps some clues can be obtained by our finding demonstrating that hydroxylamine reverses PTPase inhibition by vanadate but not by W or Mo (Table 2). This may imply that vanadate inhibits PTPase by a mechanism that is not shared with W or Mo and is more effective at the intact cell level (manuscript in preparation). It was suggested for PTPases that the hydrolysis of the phosphoenzyme intermediate (by H_2O) is the rate-limiting step in catalysis (Walton & Dixon, 1993). This probably explains the activation of PTPases by hydroxylamine (Table 2). From an enzymological standpoint, it appears that vanadate further deceases the hydrolysis of the phosphoenzyme intermediate and hydroxylamine by virtue of accelerating this rate-limiting step, reversing the inhibition.

Finally, a point which may be of future clinical significance is the observation made by us here, showing that pMo and pW are highly stable at acidic pH values as compared to pV (Figure 4). Pervanadate, when administered intraperitoneally is highly effective in inducing normoglycemia in STZ-rats (Shisheva et al., 1994) but not so effective when orally administered (unpublished observation), most likely due to the strong acidity of the stomach. Such a barrier may not exist when pW and pMo are administered orally to STZ-rats. This and other aspects raised here are currently being studied.

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