Electron Paramagnetic Resonance Studies and Insulin-like Effects of Vanadium in Rat Adipocytes†

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ABSTRACT: The entry of vanadate ions into rat adipocytes and their intracellular reduction and binding to endogenous reduced glutathione (GSH) have been detected and studied by EPR spectroscopy of the vanadyl(IV) ion. The rate of transport and reduction of vanadate was enhanced by externally added glucose, reaching a steady level after 3–4 h. Two distinct vanadyl–GSH complexes were detected within the cells with hyperfine coupling constants of 102.6 (A) and 89.3 G (B), respectively. Initially, both A and B were detected in the cells; eventually, complex B signal constituted about 90% of the total signal. EPR studies of model vanadyl–GSH complexes in aqueous solution allow identification of the stoichiometry and structure of the intracellular vanadyl–GSH complexes. These led to the following conclusions: The major vanadyl complex (B) has a 1:1 VO^{2+}:GSH stoichiometry. The vanadyl is coordinated at the equatorial positions to strong crystal field donor groups of GSH such as the thiol group of cysteine, two NH peptide groups, and the glutamyl amino group. The minor vanadyl complex (A) has also a 1:1 stoichiometry, but coordination occurs via the two carboxylate groups, the thiol group, and a water molecule. Two VO^{2+}:GSH complexes (C and D) with 1:2 stoichiometry, observed in aqueous solutions, were not detected in the cells. Previously, we have reported that vanadate fully mimics the stimulatory effect of insulin on glucose oxidation in rat adipocytes [Shechter, Y., & Karlish, S. J. D. (1980) *Nature (London) 284*, 556–558]. In this study, we show further that (a) the time course of glucose oxidation is similar if stimulated either by insulin or by vanadate, (b) vanadate and insulin stimulate $^{14}CO_2$ production via both glycolysis and the hexose monophosphate shunt, and (c) like insulin, vanadate inhibits ACTH- or isoproterenol-induced lipolysis. These observations suggest that insulin and vanadate share common steps in their mechanisms of action.

Vanadium is now recognized as an essential nutritional element in higher animals, but its function is still unclear (Frieden, 1972; Underwood, 1977). Since the discovery of vanadate as a potent inhibitor of (Na,K)-ATPase (Cantley et al., 1977), many vanadium-dependent physiological and biochemical effects have been established [for a review, see Simons (1979)]. Vanadate also inhibits other ATPases (Gibbons, 1966; Charney et al., 1975; Hudgins & Bond, 1977) and enzymes involved in phosphate transfer, such as alkaline phosphatase (Lopez et al., 1976) and phosphofructokinase (Choata & Mansour, 1978), and stimulates adenylate cyclase (Schwabe et al., 1978). Vanadate has been considered to be a possible regulator of the Na pump in vivo, but this is in doubt for vanadate(V) is reduced largely to the vanadyl form in vivo (Cantley & Aisen, 1979), and vanadyl ions may be tightly bound to proteins. Vanadyl ions are poor inhibitors of the (Na,K)-ATPase in vitro (Cantley & Aisen, 1979).

We have reported recently that externally applied vanadate ions at low concentrations mimic fully the stimulatory effect of insulin on glucose oxidation in rat adipocytes (Shechter & Karlish, 1980). We concluded that the insulin-like influence of vanadium in rat adipocytes is due to the effects of vanadyl(IV) ions, probably produced within the cells, and cannot be accounted for by inhibition of active Na^+ and K^+ transport. Dubyak & Kleinzeller (1980) have reported similar effects of vanadate on both glucose oxidation and transport of 2-deoxyglucose and confirmed that these effects are not caused by inhibition of (Na,K)-ATPase for active Rb^+ transport was unaffected by vanadate.

In continuation of the previous studies, it seemed desirable to examine whether vanadate ions enter the adipocytes and are reduced to the vanadyl state detectable by the characteristic EPR signal. In red cells, vanadate is reduced almost quantitatively to the vanadyl ion which is tightly bound to hemoglobin (Macara et al., 1980). It turns out that in the adipocytes too the vanadate is converted to vanadyl, and the VO^{2+} ions are complexed to the small intracellular peptide glutathione.

It was also of interest to ascertain further the degree of similarity in the mode of action of insulin and vanadate and establish particularly whether vanadate affects processes occurring within the cell in addition to transport functions. We have therefore looked at the effects of vanadate on glucose oxidation via both glycolysis and the hexose monophosphate shunt and examined especially whether, like insulin, vanadate inhibits lipolysis induced by isoproterenol or adrenocorticotropic hormone (ACTH).[1]

Materials and Methods

Materials. Porcine insulin was purchased from Eli Lilly Co. D-[U-^{14}C]Glucose, [1-^{14}C]glucose, and [6-^{14}C]glucose (4–7 mCi/mol) were from New England Nuclear. Collagenase type I (134 units/mg) was obtained from Worthington. All other materials used in this study were the products of Sigma or the British Drug Houses (BDH) and were of analytical grade.

Preparation of Isolated Adipocytes and Glucose Oxidation. Isolated fat cells were prepared from either Sprague-Dawley or Wistar male rats (80–120 g) according to the method of Rodbell (1964), and glucose oxidation was measured by conversion of either D-[U-^{14}C]-, [1-^{14}C]-, or [6-^{14}C]glucose to $^{14}CO_2$; 0.2 mM glucose was used.

Lipopolysis. Lipopolysis was performed by incubating the fat cell suspension at 37 °C for 3 h with isoproterenol (2 ×

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[1] Abbreviations used: KRB buffer, Krebs-Ringer bicarbonate buffer; BSA, bovine serum albumin; Cl_3CCOOH, trichloroacetic acid; GSH, reduced glutathione; ACTH, adrenocorticotropic hormone; DPPH, diphenylpicrylhydrazyl.

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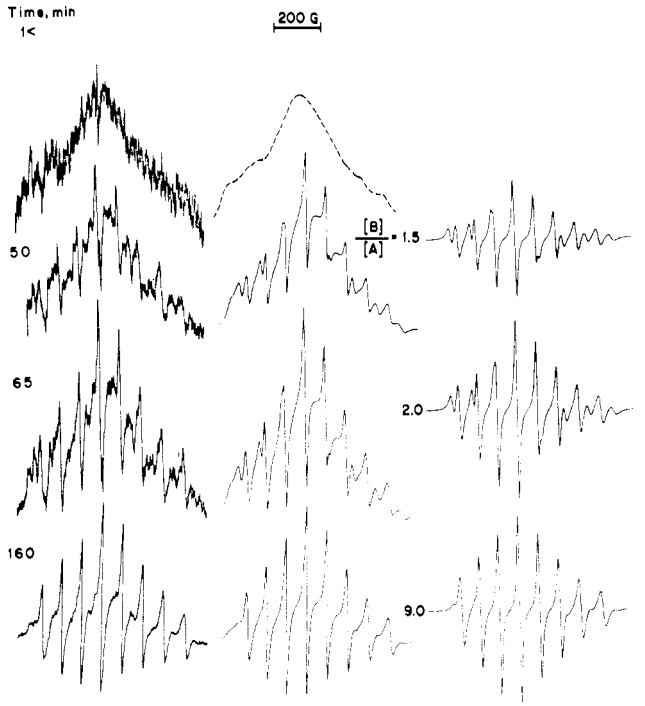


FIGURE 1: First-derivative EPR spectra of vanadyl ions in a fat cell suspension. Fat cells (about 6×10^6 cells) were incubated at room temperature in KRB buffer (pH 7.4) containing 0.1% albumin, 200 μM sodium metavanadate, and 100 mM D-glucose. Spectra were recorded at the indicated time intervals. Recording conditions are as follows: modulation frequency, 100 kHz; modulation amplitude, 10 G; microwave power, 200 mW; time constant, 3 s; scan time, 16 min; spectrometer gain for (A–C) = 1.25×10^6, for (D) = 4×10^5. DPPH was included as an internal standard (sharp peak in the middle of the spectra).

10^{-7} M) or ACTH (0.1 μg/mL) and with insulin or vanadate as mentioned in the text. Aliquots from the medium were then taken, BSA was removed by cold (6%) Cl_3CCOOH precipitation, and the glycerol content was determined by the chemical method which appeared in the *Pierce Catalogue* (1976) (triglyceride C–37 rapid stat kit).

EPR Studies. EPR spectra were recorded at room temperature with a Varian E-4 spectrometer operating in the X band, using flat quartz cells of 200-μL volume; *g* values were determined relative to DPPH as a standard for which a value of 2.00365 was taken.

Results

EPR Studies of Vanadyl Ions. Addition of sodium metavanadate (200 μM) and D-glucose (100 mM) to a suspension of fat cells resulted in the gradual appearance of EPR spectra characteristic of vanadyl ion complexes (Figure 1). No EPR signal is observed in solutions of vanadate or in suspensions of adipocytes alone. Also, by separating the cells from the external medium, it could be clearly seen that the EPR signals are associated exclusively with the cell fraction (Figure 2A), and the external medium shows no EPR signal (Figure 2B) unless 10 mM GSH is added at pH 4 to reduce the vanadate to vanadyl (Figure 2C).

In order to try and identify the vanadyl complexes formed in the cells, we have examined the EPR signals of the vanadyl ion in the presence of various low molecular weight complexants likely to be present at sufficient concentrations (e.g., ATP, NADP, and GSH).

This study led to the conclusion that in adipocytes the vanadyl ion is complexed to GSH. Figure 3 shows four spectra observed in solutions containing variable amounts of vanadyl

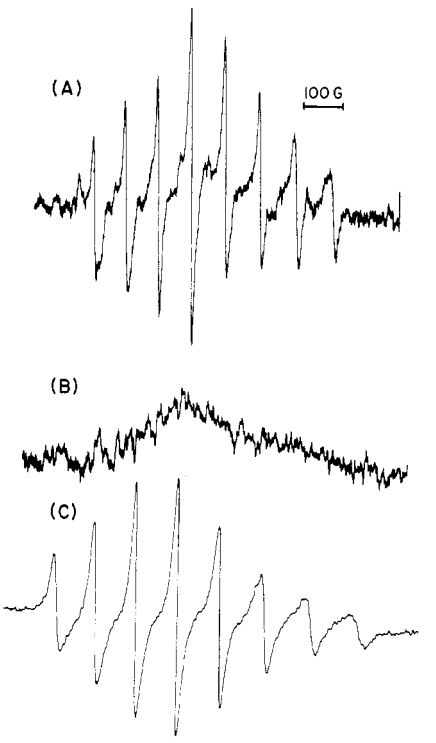


FIGURE 2: EPR spectrum of VO^{2+} within the fat cell and its absence in the external medium. Fat cells were incubated for 90 min at room temperature with $NaVO_3$ (1 mM) and glucose (100 mM). The medium was then separated from the cells, and both fractions were examined for their EPR spectra. EPR spectrum of the fat cell fraction (A) and that of the external medium (B). EPR spectrum of the external medium after its reduction with GSH (10 mM) at pH 4.0 (C). Recording conditions are as follows: modulation amplitude, 10 G; microwave power, 125 mW in (A) and (B) and 100 mW in (C); time constant, 1 s; scan time, 16 min; spectrometer gain, 6.2×10^5 in (A) and (B) and 1.25×10^5 in (C).

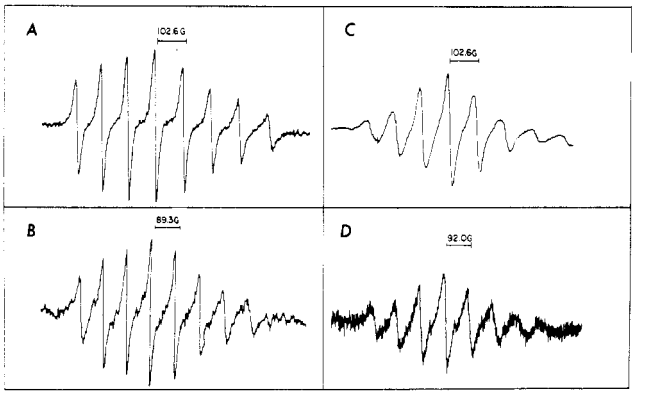


FIGURE 3: EPR spectra of the various glutathione complexes of vanadyl. The value, in gauss, of the isotropic hyperfine coupling constant of each complex is indicated in the figure. (A) A solution of 8 mM $NaVO_3$ and 25 mM GSH at pH 5. (B) A solution of 0.7 mM $NaVO_3$ and 2.7 mM GSH at pH 7. (C) A solution of 5 mM $VOSO_4$ and 100 mM GSH at pH 5.5. (D) Same solution as in (C) at pH 8.0. All solutions were prepared and measured under nitrogen. Recording conditions were as follows: modulation amplitude for (A), (C), and (D) = 10 G and for (B) = 12.5 G; microwave power, (A) = 25 mW, (B) = 150 mW, (C and D) = 100 mW; spectrometer gain, (A) 1.5×10^5, (B) 3.2×10^5, (C) 4×10^4, (D) 4×10^5.

and GSH and at different pHs. Two complexes formed at acidic pH, A and C (Figure 3), were observed at a GSH:VO^{2+} concentration ratio of 3 and 20, respectively. They have the same isotropic hyperfine coupling constant (Aiso) of 102.6 G but differ in their line width. The other two (B and D) were observed in neutral to slightly basic solutions at a GSH:VO^{2+} concentration ratio of 4 and 20, respectively. They differ too

Table I: EPR Parameters of VO^{2+}–GSH Complexes

complex	pH	[vanadyl] (mM)	[GSH] (mM)	g	Aiso (G)	relaxation parameters (G) a_1	a_2	a_3	equatorial ligands	calcd Aiso (G)
A	5	8[a]	25		102.6	12.8	1.3	0.6	$-S^-$; $-COO^-$; $-COO^-$; H_2O	100–102
B	7	0.7[a]	2.7	1.9629	89.3	9.0	0.8	0.6	$-S^-$; -NH-; -NH-; $-NH_2$	86–90
C	5.5	5	100		102.6	22.7	3.9	2.8		
D	8	5	100		92.0	17.3	2.3	1.5		

[a] Vanadate was added and then reduced by GSH under nitrogen to vanadyl.

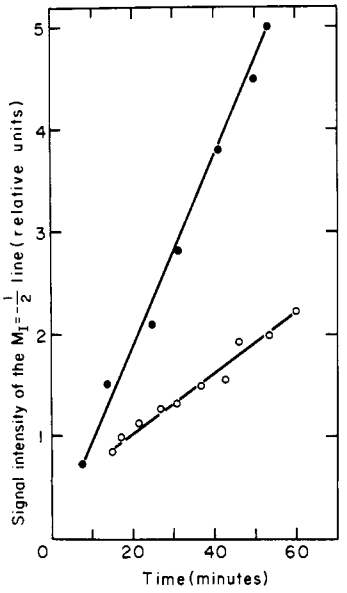


FIGURE 4: Time course for the appearance of the VO^{2+} signal in adipocytes; effect of glucose. Fat cells (about 6×10^6 cells) were incubated in KRB buffer (pH 7.4) which contains 8 mM sodium metavanadate ($NaVO_3$) with (●) or without (○) D-glucose (100 mM). At different time intervals, the EPR signal intensity of the $m_I = {}^1/_2$ line of VO^{2+} was recorded. The intensities recorded are relative to those initially measured for each experiment.

in line width but have a similar and remarkably small Aiso, 89.3 and 92.0 G for B and D, respectively. The line widths of each complex were found to depend parabolically on the vanadyl quantum number (m_I), the width of each hyperfine component being given by the expression $1/[T_2(m_I)] = a_1 + a_2 m_I + a_3 m_I^2$. The derivation of the constants a_1, a_2, and a_3 for an axial symmetric complex of vanadyl can be obtained from Kivelson's theory and has been given explicitly by Rogers & Pake (1960). Table I lists the relaxation constants for each complex. These constants were calculated from the best fit of the width of the eight hyperfine components to the above equation.

Computer simulations of the spectra recorded in cells using the EPR parameters obtained in vitro (Figure 1) led to the conclusion that the major and minor intracellular complexes have the same EPR constants as complexes B and C, respectively (Figure 3). The concentration ratio of B to A was found to increase with time as shown in Figure 1.

In a glucose-free medium, the intensity of the vanadyl signal in the fat cells (measured as the height of the fourth transition) increased with time (Figures 1 and 4), and the rate of formation of the vanadyl ion was accelerated 2–3 fold in the presence of externally added glucose (Figure 4). With added glucose, a steady level was reached after about 4 h of incubation.

Effects of Vanadate on Glucose Oxidation. The following experiments provide further evidence concerning penetration of vanadate into the adipocytes and its consequences for glucose oxidation. The time course of glucose oxidation was

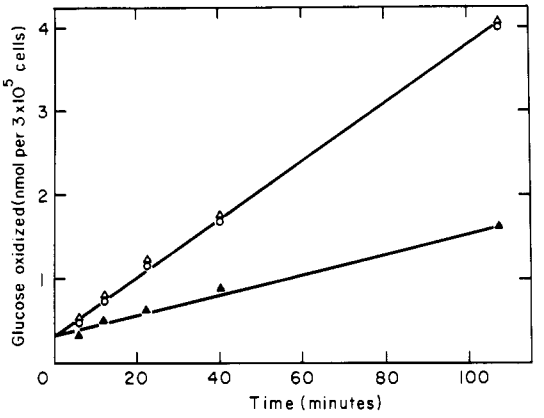


FIGURE 5: Time course of glucose oxidation by insulin and vanadate. Isolated adipocytes were incubated for 15 min at room temperature with either insulin (1.67×10^{-8} M, ▲), sodium orthovanadate (200 μM, ○), or neither (△). The cells were then incubated at 37 °C with 0.2 mM D-[U-^{14}C]glucose. At the indicated time intervals, samples were withdrawn, and the amount of glucose oxidized was determined.

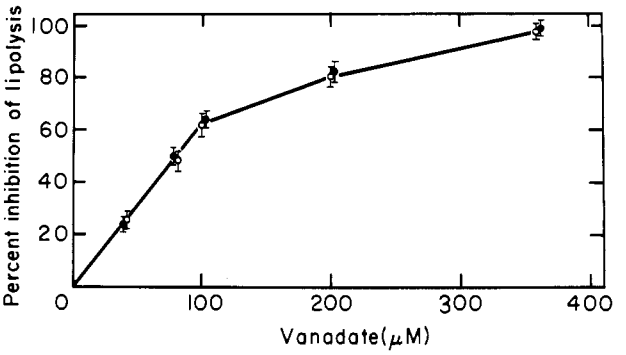


FIGURE 6: Effect of vanadate on inhibition of lipolysis. Fat cells (about 1×10^5 cells) in KRB buffer (pH 7.4) were incubated for 3 h at 37 °C with either isoproterenol (2×10^{-7} M) or ACTH (2 μg/mL) and varying concentrations of $NaVO_3$ (○) or $NaVO_3$ and GSH (1 mM, ●). Cells were then removed, and the amount of glycerol released to the medium was determined. This amounted to 60 and 200 nmol in the absence and the presence of isoproterenol, respectively. Vanadate alone did not have any lipolytic effect. Insulin (1–10 nM) inhibited 60–80% of the isoproterenol-mediated glycerol release in this system.

similar if stimulated either by insulin or by 200 μM vanadate (Figure 5). In neither case was there a measurable time lag prior to maximal stimulation of glucose oxidation. This indicates that the concentration of the vanadyl ions within the cells at the shortest time, i.e., 6 min, is sufficient to produce a maximal stimulation, and entry of vanadate and reduction to the vanadyl state do not limit the rate of glucose oxidation. Vanadate resembles insulin in its ability to stimulate $^{14}CO_2$ production via both the Embden-Meyerhof glycolytic pathway (from [6-^{14}C]glucose) and the hexose monophosphate shunt (from [1-^{14}C]glucose, Table I).

Antilipolysis. Figure 6 demonstrates that, like insulin, vanadate inhibits isoproterenol- or ACTH-stimulated lipolysis. The extent of the effect was usually greater with vanadate than with insulin, 100% inhibition being observed with vanadate

compared with a maximal extent of inhibition by insulin of 60–80%. Fifty percent inhibition was observed at about 80 μM vanadate, a concentration about 4 times higher than that required for half-maximal stimulation of glucose oxidation (Shechter & Karlish, 1980). Addition of 1 mM GSH, which was found previously to lower the $K_{0.5}$ for vanadate-mediated stimulation of glucose oxidation by almost 1 order of magnitude (Shechter & Karlish, 1980), did not improve inhibition of lipolysis by submaximal concentrations of vanadate.

Discussion

The results presented show that extracellular vanadate ions permeate into the adipocyte cells and are then reduced to vanadyl ions. The vanadyl ions interact with the intracellular GSH to form two kinds of 1:1 VO^{2+}:GSH complexes. The two complexes assigned A and B were identified by comparing their EPR parameters with those found in solutions of vanadyl and GSH. The major complex (particularly at steady state) has the same isotropic hyperfine coupling constant and relaxation constants as complex B (Figure 3, Table I) formed in a solution similar in vanadate and GSH concentration, and in pH, to that found in the intracellular medium. The second complex, the concentration of which at steady state is about one-tenth of that of the major complex, has the same EPR constants as complex A (Figure 3, Table I) observed as the sole EPR signals at pH 5.0. The relative amount of the two complexes within the cells, [B]/[A], varies with time, as shown in Figure 1. It is conceivable that at the initial stages of the transport reduction process the internal pH is more acid than normal so some complex A can be formed, but then the pH returns to the physiological value (7.2–7.4) at the steady state when complex B predominates. We are trying now to test the prediction of these pH changes by other methods.

The detailed structure of the various GSH complexes with glutathione is still being investigated. However, from the EPR studies, some structural conclusions can already be drawn. The complexes (A–D) differ in either their isotropic hyperfine coupling constant (Aiso) or their relaxation constants (a_1, a_2, and a_3) (see Figure 3 and Table I). The differences in Aiso are pH dependent and reflect primarily a change in the nature of the ligating groups. The relaxation constants depend on the concentration of reactants and, for complexes having similar hyperfine constants, reflect mainly changes in the correlation time for rotation which in turn is determined by the size of the complex. The two acidic complexes A and C (Figure 3) have the same isotropic hyperfine coupling constant, Aiso = 102.6 G, indicating that coordination is similar in both complexes. However, their relaxation constants differ by a factor of >2, suggesting a stoichiometry of 1:1 and 1:2 VO:GSH for A and C, respectively. The two basic complexes B and D (Figure 3) have similar and remarkably small hyperfine coupling constants (Aiso = 89.3 G for B and Aiso = 92.0 G for D), thus indicating participation of similar ligands but a major change in coordinating groups must have occurred by comparison with the acidic complexes. The relaxation constants of B and D also differ by a factor >2, also suggesting a stoichiometry of 1:1 and 1:2 VO^{2+}:GSH, respectively.

From values of isotropic hyperfine coupling constants of vanadyl complexes, it is possible to predict empirically the nature of the complexing ligands. Wüthrich (1965a,b) showed that for some VO^{2+} complexes isotropic hyperfine coupling reflects the average environment around the vanadyl ion contributed by the four equatorial ligands, so that one can predict the experimental value of the Aiso by the following equation:

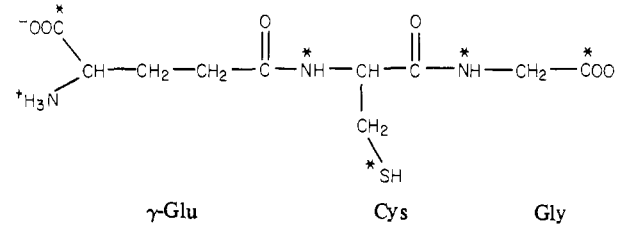
$$\text{Aiso} = (A_{L1} + A_{L2} + A_{L3} + A_{L4})/4$$

Table II: Hyperfine Coupling Constants (A_L) of Vanadyl Complexes Having Four Identical L Ligands in the Equatorial Positions

L	A_L (G)	model complexes	reference
$-COO^-$	102	$[VO(malonate)_2]^{2-}$	Wüthrich (1965b)
-SH	82–86[a]	VO^{2+} complexes of monothio-β-diketones	Bozis & McCormick (1970)
		VO^{2+} complexes of β-diketones	McCormick & Bellott (1970)
$-NH_2$	88–96[a]	dimethylalanine crystals	Tomkiewicz et al. (1972)
-NH		VO^{2+} porphyrin	Kivelson & Lee (1966)

[a] Limits calculated from data on mixed compounds, using Wütrich formula.

A_L is the isotropic coupling constant found experimientally for a vanadyl complex that had four identical ligands of a particular type bonded to the VO^{2+} in the equatorial positions. Thus, binding to weak crystal-field ligands will increase Aiso, while binding to strong crystal-field ligands will reduce it. Previous studies of GSH binding to metal ions indicated that GSH is a multidentate chelate [see review of Rabenstein et al. (1979)]. It can form complexes which differ in their stoichiometry and possesses various donor sites as indicated by asterisks in the following formula for glutathione:



Using Wüthrich's empirical formula, we are able to suggest the ligands binding in the 1:1 VO^{2+}:GSH complexes, as given in Table I. The A_L for each donor group of GSH was estimated from the data in Table II. For the 1:2 complexes, several structures can be constructed to fit with the experimental Aiso values, and definite conclusions concerning coordination sites cannot be drawn.

In vivo reduction of vanadate(V) to vanadyl(IV) in red blood cells has been suggested by Macara et al. (1980) to occur via cytoplasmic GSH. This reduction was nearly quantitative after 23 h. The investigators proposed that the reduction may be nonenzymatic, since addition of GSH and hemoglobin to VO^{2+} ions in vitro produced a pattern of reduction and binding of VO^{2+} to hemoglobin similar to that observed with the cells. In our studies, the EPR signal reached a steady level after about 3–4 h, and the rate of reduction was enhanced in the presence of glucose. It is possible that the reduction of vanadate by GSH is purely chemical, and the function of the glucose is to produce NADH, which maintains the level of reduced glutathione via the glutathione reductase reaction. However, it is also possible that vanadate is reduced by NADH by an enzymatic process recently reported to exist in cardiac cell membranes (Erdmann et al., 1979).

Previously, we have proposed that the insulin-like effect of vanadate may involve inhibition of protein phosphatase activity which controls the level of phosphorylation of protein(s) involved in glucose transport and/or oxidation. It is of interest in this connection that Dubyak & Kleinzeller (1980) have reported that vanadate stimulation of glucose transport, like that of insulin, requires cellular ATP. Alternatively, and in view of the involvement of GSH in reducing vanadate, one

Table III: Oxidation of [6-^{14}C]- and [1-^{14}C]Glucose by Adipocytes; Effect of Insulin and Vanadate[a]

addition	nmol of glucose oxidized per 2 h	
	from [1-^{14}C]glucose	from [6-^{14}C]glucose
none	2.5 ± 0.1	1.53 ± 0.07
insulin (0.67×10^{-7} M)	6.05 ± 0.2	5.24 ± 0.12
vanadate (200 μM)[b]	4.9 ± 0.1	4.96 ± 0.05

[a] Isolated fat cells were prepared from Wistar rats (90–100 g). The final reaction volume of 0.5 mL contained 0.2 mM glucose and about 3×10^5 cells suspended in KRB buffer (pH 7.4). [b] Sodium metavanadate ($NaVO_3$) was applied.

could propose that the effects of vanadate are related to the intracellular redox potential (controlled by the GSH levels), by a mechanism analogous to that suggested by Mukherjee et al. (1978) and Mukherjee & Lynn (1977, 1979), to explain insulin-like effects of sulfhydryl oxidants. These authors propose that insulin induces a peroxidative metabolism coupled to GSH oxidation which leads finally to an increase in cellular NADP and a decrease in NADPH and accelerated flux through the hexose monophosphate shunt. This particular mechanism might explain stimulation by vanadate of glucose oxidation via the shunt path, but it does not explain the other insulin-like effects of vanadate, namely, activation of the hexose transport system (Dubyak & Kleinzeller, 1980), stimulation of glucose oxidation via the Embden-Meyerhof path (Table III), or inhibition of lipolysis (Figure 6). Nevertheless, in view of the identification of two GSH:VO^{2+} complexes, it is possible that one or both of these are the effective species rather than free VO^{2+} ions.

The other results in this paper provide further evidence for the similarity in the actions of insulin and vanadate. These include the identical time courses of glucose oxidation, stimulation of $^{14}CO_2$ production via both glycolysis and the hexose monophosphate shunt, and the antilipolytic activity. It is, therefore, reasonable to assume that activation by insulin or vanadate of the glucose transport at the cell membrane and antilipolysis within the cell share common steps or a common signal. In addition to the overall similarity in the effects of vanadate on glucose oxidation and antilipolysis, there are also detailed differences, notably a requirement for higher vanadate concentrations and lack of stimulation by externally added GSH on the antilipolytic activity. Firm evidence for the mechanism of action of insulin and vanadate, particularly of the common steps, will require a detailed investigation of the processes affected at the subcellular level.

Acknowledgments

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