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Oxovanadium(IV)–Porphyrin Complex as a Potent Insulin-Mimetic. Treatment of Experimental Type 1 Diabetic Mice by the Complex [*meso*-Tetrakis(4-sulfonatophenyl)porphyrinato]oxovanadate(IV)(4−)

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We prepared and characterized [*meso*-tetrakis(4-sulfonatophenyl)porphyrinato]oxovanadate(IV)(4−), [VO(tpps)], and its in vitro insulin-mimetic activity, metallokinetic features in the blood of healthy rats, and in vivo hypoglycemic effect in streptozotocin (STZ)-induced diabetic mice (STZ mice) were investigated. The results were compared with those of previously proposed insulin-mimetic [*meso*-tetrakis(1-methylpyridinium-4-yl)porphyrinato]oxovanadium(IV)(4+), [VO(tmpyp)], and vanadium(IV) oxide sulfate. The in vitro insulin-mimetic activity, the retention time and bioavailability of [VO(tpps)] in blood were considerably better than those of [VO(tmpyp)] and vanadium(IV) oxide sulfate. [VO(tpps)] caused a significant hypoglycemic effect in STZ mice within 8 h following a single oral administration of the complex at 15 mg V/kg of body mass without ascorbate; this effect was sustained for at least 60 h. [VO(tpps)] normalized the hyperglycemia of STZ mice within 2 days when administered orally at 4–10 mg V/kg of body mass for 18 days. Vanadium, as determined by instrumental neutron activation analysis, was distributed in the tissues examined in the following decreasing order: bones, kidneys, liver, lungs, spleen, heart, pancreas, muscles, fatty pads, and brain. The improvement in diabetes was supported by oral glucose tolerance test, HbA_{1c} level and blood pressure. Based on the above results, [VO(tpps)] is an orally active oxovanadium(IV)–porphyrin complex for treating type 1 diabetic animals.

Diabetes mellitus (DM) is a group of important metabolic syndromes that results from an absolute, or relative, deficiency of insulin secretion and/or its action, and it is widely accepted as one of the leading causes of death and disability worldwide. It can affect nearly every organ system in the body and lead to blindness, end-stage renal disease, lower extremity amputations, increased risk of stroke, ischaemic heart disease, peripheral vascular disease and neuropathy. Based on its type, the treatment for DM involves either daily injections of exogenous insulin (most common for type 1, i.e., insulin-dependent DM) or oral administration of hypoglycemic drugs, such as sulfonylureas,[1] metformin,[2] alpha-glucosidase inhibitors, thiazolidinediones, and meglitinides,[3] and in a combination therapy for type 2 (non-insulin-dependent) DM.[4] However, this approach is not satisfactory for a large proportion of patients; hence, there have been continued efforts towards developing new hypoglycemic drugs with high potency, but little or no side effects. It has been established that vanadium compounds exert an insulin-mimetic action in both in vitro and in vivo systems, including their ability to improve glucose homeostasis and insulin resistance in animal models of DM.[5–7] In recent years, several reports have documented vanadium therapy-induced improvements in insulin sensitivity in the liver and muscles of many type 2 diabetic human subjects.[8–10]

Since the discovery of the orally active insulin-mimetic oxovanadium(IV)–cysteine methyl ester complex for the treatment of streptozotocin (STZ)-induced diabetic rats in 1990,[11] the therapeutic potential of oxovanadium(IV) complexes has been of great interest to us. We and other researchers have developed several types of oxovanadium(IV) complexes with different vanadium coordination spheres, such as $VO(N_2S_2)$, $VO(N_2O_2)$, $VO(S_2O_2)$, $VO(O_4)$, and $VO(S_4)$.[12–20] These complexes showed normoglycemic activity in type 1 and type 2 DM animals. However, only a few complexes, such as bis(biguanidato)oxovanadium(IV), $[VO(big)_2]$, bis(*N*′,*N*′-dimethylbiguanidato)oxovanadium(IV), $[VO(metf)_2]$, and bis(β-phenethylbiguanidato)oxovanadium(IV), $[VO(phenf)_2]$, with a $VO(N_4)$ vanadium coordination sphere, have been examined.[21] Among these, $[VO(metf)_2]$ was found to significantly lower the blood glucose levels in STZ-induced diabetic rats—a type 1 diabetic model—by oral gavage. However, hypoglycemic activity was sustained for 24 h. Recently, we found that [*meso*-tetrakis(1-methylpyridinium-4-yl)porphyrinato]oxovanadium(IV)(4+), [VO(tmpyp)], (Fig. 1a), in which the vanadium coordination sphere is $VO(N_4)$, is a potential insulin-mimetic oxovanadium(IV)–porphyrin complex for the treatment of STZ rats by intraperitoneal (*i.p.*) injection together with sodium ascorbate.[22] This important finding prompted us to devel-

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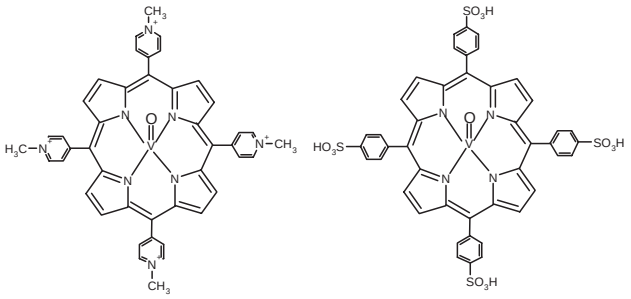

Fig. 1. Structures of [VO(tmpyp)] (a) and [VO(tpps)] (b).

op more active oxovanadium(IV)–porphyrin complexes. The insulin-mimetic activity of [*meso*-tetrakis(4-sulfonatophenyl)-porphyrinato]oxovanadate(IV)(4−), [VO(tpps)], (Fig. 1b), after a single oral administration to STZ mice,[23] has opened up the possibility of estimating its detailed in vivo normoglycemic activity in the STZ mice. Here, we report the complete synthesis of [VO(tpps)] and its stability in 4% bovine serum albumin, as well as in the fresh blood serum of rats, in the absence of ascorbate. In addition, the in vitro insulin-mimetic activity in the absence and presence of ascorbate, metallokinetic analysis in the blood of healthy rats and in vivo normoglycemic activity, when orally administered to the STZ mice are reported. Finally, we compare the above results with those of [VO(tmpyp)] and vanadium(IV) oxide sulfate as controls.

Experimental

Materials. All reagents and solvents were commercially available and of the highest grade of purity; hence, they were used without purification. Vanadium(IV) oxide sulfate, $VOSO_4 \cdot nH_2O$, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The ligands *meso*-tetrakis(1-methylpyridinium-4-yl)porphyrin, H_2tmpyp, and *meso*-tetrakis(4-sulfonatophenyl)porphyrin, H_2tpps, were purchased from Frontier Scientific Inc. (P.O. Box 31, Logan, UT, U.S.A.). $VOSO_4 \cdot nH_2O$ was standardized complexometrically with ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), determined to be the trihydrate, and used in all of the experiments. Sephadex LH-20 was obtained from Amersham Pharmacia Biotech (Tokyo, Japan). Bovine serum albumin (fraction V), (±)-epinephrine monohydrochloride, STZ and collagenase were purchased from Sigma Chemical (St. Louis, MO, U.S.A.).

Syntheses of the Complexes. The oxovanadium(IV) derivative of H_2tpps was prepared according to the method used by Adler et al.[24] with slight modifications. [VO(tmpyp)] was synthesized as described previously.[22]

[VO(tpps)]: H_2tpps (0.2 g, 0.2 mmol) and vanadium(IV) oxide sulfate ($VOSO_4 \cdot 3H_2O$, 0.86 g, 3.99 mmol) were heated to reflux in *N*,*N*-dimethylformamide (DMF; 50 mL) on a stirring hot plate at 150 °C for 24 h. The refluxing solution was then evaporated to approximately 5 mL and cooled in an ice bath. Acetone (20 mL) was added to the remaining solution. The resulting precipitate was redissolved in methanol and reprecipitated with acetone six times. The crude material was purified by gel chromatography (Sephadex LH-20; eluent: H_2O). Finally, the aqueous solution was concentrated and dried under high vacuum. The composition of the complex was determined by elemental analyses, mass spectrometry, IR, and ESR spectra (Table 1), and then compared with the data from relevant literature.[25]

[VO(tmpyp)]: H_2tmpyp (546 mg, 0.4 mmol) and vanadium(IV) oxide sulfate ($VOSO_4 \cdot 3H_2O$, 600 mg, 1.6 mmol) were heated to reflux in Millipore distilled water (H_2O; 50 mL) on a stirring hot plate at 130 °C for 48 h. This solution was cooled and separated from a trace amount of insoluble material by filtration, and 10 g of $NaClO_4$ was added, after which a dark precipitate formed. The mixture was chilled overnight, and the solid was isolated by filtration and washed 6–7 times with dilute (2%) perchloric acid to remove excess metal ions. Finally, the solid product was dried under high vacuum. The composition of the complex was determined by elemental analyses, IR, and ESR spectra (Table 1), and then compared with our previous data.[22]

Physical Measurements. Elemental analyses were carried out using a Perkin-Elmer 240C elemental analyzer (Wellesley, MA, U.S.A.). IR spectra of the solid samples in compressed KBr disks were measured using a Shimadzu FTIR-8100A spectrophotometer (Shimadzu, Kyoto, Japan). UV–visible absorption spectra in aqueous solvents were recorded with an Agilent 8453 UV–visible spectrometer (Agilent, Germany). Low resolution mass spectra were recorded with a JEOL JMS-SX 102AQQ spectrometer (JEOL, Tokyo, Japan) in FAB(−) mode by using methanol, or 3-nitrobenzyl alcohol, as the matrix material; this was performed at the Analytical Center of Kyoto Pharmaceutical University. ESR spectra were measured using a 1.0 mM solution of the complexes at both room temperature (22 °C) and liquid nitrogen temperature (77 K) using of an X-band ESR spectrometer (JES RE1X, JEOL, Tokyo, Japan) under the following conditions: frequency, 9.4 GHz; microwave power, 5.0 mW; modulation frequency, 100 kHz; mod-

Table 1. Physicochemical Properties of Oxovanadium(IV)–Porphyrin Complexes

Complex	IR spectrum in KBr $\nu_{V=O}/cm^{-1}$	Elemental analysis Calcd. (Found)		
		C%	H%	N%
$C_{44}H_{28}O_{12}S_4N_4VO \cdot 8H_2O \cdot 2C_3H_7NO$ [VO(tpps)]	1005	46.55 (46.21)	4.53 (4.48)	6.51 (6.55)
$C_{44}H_{36}N_8VO(ClO_4)_4 \cdot 5H_2O$ [VO(tmpyp)]	1007	42.91 (42.86)	3.76 (3.44)	9.10 (9.46)

Complex	Visible absorption spectrum			Solvent	ESR parameter					
					g-value			*A*-value/10^{-4} cm^{-1}		
	λ_1/nm (ε)[a]	λ_2/nm (ε)[a]	λ_3/nm (ε)[a]		g_0	$g_{//}$	$g_\perp$	A_0	$A_{//}$	$A_\perp$
[VO(tpps)]	436 (219.5)	564 (18.8)	604 (7.3)	H_2O	1.967	1.938	1.982	106	183	67
[VO(tmpyp)]	439 (190.0)	563 (15.0)	603 (3.4)	H_2O	1.982	1.966	1.990	78	160	37

a) 10^3 M^{-1} cm^{-1}.

ulation amplitude width, 0.63 mT; response, 0.03 s; scanning time, 4 min; magnetic field, 340 ± 100 mT; standards, tetracyanoquinodimethane lithium salt (TCNQ-Li) ($g = 2.00252$) and Mn(II) in MgO (magnetic field between the third and fourth signals due to Mn(II), 8.69 mT). The hyperfine coupling constant (A_0), which was estimated as the magnetic field between the $M_I = -1/2$ and 1/2 hyperfine components, and g_0 value were obtained from the spectra at room temperature. $A_{//}$, which was estimated as the mean on the basis of the spectral regions for the $M_I = -5/2$ and $-7/2$ (A^1) and $M_I = 5/2$ and 7/2 (A^2) hyperfine components, and $g_{//}$ values were obtained from the spectra at 77 K. The $g_\perp$ and $A_\perp$ values were obtained using the following equations:

$$g_\perp = 1/2(3g_0 - g_{//}). \quad (1)$$

$$A_\perp = 1/2(3A_0 - A_{//}). \quad (2)$$

Stability of Oxovanadium(IV)–Porphyrin Complexes in Serum Solutions. The oxovanadium(IV)–porphyrin complexes ([VO(tmpyp)]: 1 mM; [VO(tpps)]: 1 mM) were mixed with 4% bovine serum albumin in saline solution (2 mL) in a stoppered sample tube which was then shaken in a water bath at 37 °C for 48 h. The oxovanadium(IV) state of the porphyrin complexes in 4% bovine serum albumin was monitored by ESR at room temperature by withdrawing a small aliquot (200 μL) from the solution. The stability of the oxovanadium(IV) complexes was also studied in the fresh blood serum of rats, which was used instead of 4% bovine serum albumin. The fresh blood serum was collected from the supernatant of normal Wistar rat blood centrifuged at 3000 rpm for 10 min. The oxovanadium(IV) species were also monitored by ESR in the same manner as described above.

Evaluation of In Vitro Insulin-Mimetic Activity. The insulin-mimetic activity of [VO(tpps)] was evaluated by simultaneous in vitro experiments, in which both the inhibitory activity upon release of free fatty acid[26] and the enhancement of glucose uptake ability[27] of the complex in isolated rat adipocytes treated with epinephrine were estimated and compared with those of [VO(tmpyp)] and vanadium(IV) oxide sulfate as positive controls. Male Wistar rats (weighing 200 g) were sacrificed under anesthesia with ether. The adipose tissues were removed, chopped with scissors and digested with collagenase for 1 h at 37 °C in Krebs Ringer bicarbonate buffer, pH 7.4, containing 2% bovine serum albumin. The adipocytes, thus obtained, were then separated from undigested tissues by filtration through a nylon mesh (250 μm) and washed three times with the buffer in the absence of collagenase. The complex was dissolved in saline at various concentrations. 30 μL of each solution and 10 μL of glucose (final concentration: 5 mM) along with either 5 μL of H_2O or 5 μL of sodium ascorbate (final concentration: 1 mM) were added to 240 μL of the isolated adipocytes (1×10^6 cells mL^{-1}), and the resulting suspensions were incubated at 37 °C for 30 min. Finally, 15 μL of epinephrine (final concentration: 10 μM) was added to the suspensions, and the resulting mixture was incubated at 37 °C for 3 h. The reaction was stopped by cooling in ice water, and the mixtures were centrifuged at 3000 rpm at 4 °C for 10 min. The free fatty acid concentration in the outer solution of the cells was determined with a free fatty acid kit (NEFA C-test Wako, Wako Pure Chemical Industries, Osaka, Japan). The IC_{50} value, i.e., the 50% inhibitory concentration of the complex, was determined from the curve for the concentration-dependent inhibitory effect of the complex on free fatty acid release in isolated rat adipocytes treated with epinephrine in the absence, as well as in the presence, of sodium ascorbate. In addition, the glucose concentration in the outer solution of the cells was estimated using a Fuji Dry Chem analyzer (Fuji Medical Co., Tokyo, Japan). The glucose uptake ability of the compounds was evaluated using the apparent EC_{50} values, which are the 50% enhancing concentration of the compound with respect to the maximal glucose uptake concentration in epinephrine-treated adipocytes in the absence and presence of sodium ascorbate.

Metallokinetic Analysis of Oxovanadium(IV)–Porphyrin Complexes by Blood Circulation Monitoring-ESR. The metallokinetic features of the oxovanadium(IV) complexes in the blood of healthy rats that received doses (0.5 mg of V/kg body mass) of [VO(tmpyp)], [VO(tpps)], and vanadium(IV) oxide sulfate were analyzed by in vivo blood circulation monitoring-ESR.[28–30] In short, the rats were anaesthetized by *i.p.* injection of pentobarbital and maintained at 35 °C on a Deltaphase Isothermal Pad (Model 39 DP, Braintree Scientific, MA, U.S.A.). Heparinized polyethylene tubes were cannulated into the left femoral artery and vein. The free ends of the cannula were joined with heparinized silicon tubes to form a blood circuit outside the body; this was directly connected to an ESR cell (a quartz 20-μL capillary tube). Blood from the femoral artery was returned to the femoral vein and recirculated after flowing through the ESR cell by the rat's own heartbeat and blood pressure without depletion. [VO(tmpyp)] and [VO(tpps)] in saline solutions were administered to the rats by a single *i.v.* injection, and the ESR spectra were recorded at room temperature every 30 s using an X-band ESR spectrometer. Data were collected and analyzed on a Windows computer using WIN-RAD Data Analyzer (Radical Research, Tokyo, Japan). The disappearance of the ESR signal due to the oxovanadium(IV) species in the blood was plotted against time following the administration of [VO(tmpyp)] and [VO(tpps)]. To determine the concentrations of the oxovanadium(IV) species, 20 μL of various concentrations of [VO(tmpyp)] and [VO(tpps)] dissolved in the blood of untreated rats was added to the ESR cell. The samples were freshly prepared by using fresh blood spiked with each complex and, each calibration curve was obtained by monitoring the signal intensities of the central peak due to the corresponding oxovanadium(IV) complex. Metallokinetic parameters for oxovanadium(IV) complexes were obtained on the basis of a one-compartment model. Using the nonlinear least-squares regression program MULTI[28–30] that was rewritten with Visual Basic, the individual profiles of the determined concentrations of oxovanadium(IV) complexes in the blood of rats that were injected with [VO(tmpyp)] or [VO(tpps)] were fitted to the equation $[C_b = D/V_d \exp(-k_e t)]$, where C_b is the blood concentration, D is the dose of a compound, V_d is the distribution volume, k_e is the elimination rate constant, and t is the time. The area under the concentration curve (AUC), mean resistance time (MRT), total clearance (CL_{tot}), and half life ($t_{1/2}$) were calculated from the following equations: $AUC = D/V_d/k_e$, $MRT = 1/k_e$, $CL_{tot} = V_d \cdot k_e$, and $t_{1/2} = 0.693/k_e$.

Evaluation of In Vivo Antidiabetic Activity. Diabetes was induced in 6-week-old male Std: ddY mice, weighing approximately 30 g, by two *i.p.* injections of freshly prepared STZ (100 mg/kg of body mass) in 0.1 M citrate buffer (pH 5). Blood samples for the analysis of glucose levels were collected from the tail veins of the STZ mice, and the blood glucose levels were measured by using the glucose oxidase method using a Glucocard (Arkray, Kyoto, Japan). The STZ mice with a blood glucose level of 450–550 mg dL^{-1} (25–30.6 mM) 4 weeks after the first STZ administration were used for the experiments. The in vivo antidiabetic activity of the oxovanadium(IV)–porphyrin complexes was assessed in the STZ mice using both a single oral gavage and a daily oral administration of the complex for 18 days. The changes in the blood glucose level of the STZ mice after a single oral gav-

age of [VO(tmpyp)] and [VO(tpps)] at a dose of 15 mg of V/kg of body mass without ascorbate and the ligand H_2tpps at a dose of equimolar concentration of [VO(tpps)] were monitored. The complexes and ligand were dissolved in saline. For the daily oral administration, the STZ mice were treated with saline alone, or [VO(tpps)] in saline, at doses in the range of 4–10 mg of V/kg of body mass for 18 days. The blood sample for the daily analyses of glucose levels was collected from the tail vein of each mouse, and the blood glucose level was measured using a Glucocard. The body mass of mice, which were allowed free access to solid food (MF, Oriental Yeast, Tokyo, Japan) and tap water, were measured daily before the administration of saline and [VO(tpps)]. Moreover, the intake of solid food and drinking water of each mouse was checked daily throughout the experiment.

After the daily oral administration of saline alone, or [VO(tpps)] in saline, for 18 days, blood samples were collected by orbital exsanguination from the mice anaesthetized with ether and were centrifuged at 5000 rpm for 10 min at 4 °C. The serum samples were separated and analyzed for urea nitrogen, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, triglycerides, total cholesterol, free fatty acid, and insulin levels. The serum urea nitrogen, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, triglycerides, and total cholesterol levels were estimated by using a Fuji Dry Chem analyzer (Fuji Medical Co., Tokyo, Japan). The NEFA C-test and Glazyme insulin-EIA test (Wako Pure Chemical Industries, Osaka, Japan) were used to determine the serum free fatty acid and insulin levels, respectively. Moreover, the glycosylated hemoglobin (HbA_{1c}) levels in the blood collected from the tail veins of the mice were measured by using a DCA 2000 system (Bayer, Tokyo, Japan).

Oral Glucose Tolerance Test. After the daily administration of the saline alone, or [VO(tpps)] in saline, for 18 days, an oral glucose tolerance test was performed. The STZ mice were fasted for 12 h, and glucose at a dose of 1 g/kg body mass was administered orally. Blood samples were collected from the tail veins at 0, 15, 30, 45, 60, 90, 120, and 180 min after glucose administration. The blood glucose levels were measured using Glucocard.

Measurement of Systolic Blood Pressure. Using a Model MK-2000 BP monitor for rats and mice (Muromachi Kikai, Tokyo, Japan) according to the manufacturer's instructions, the systolic blood pressure of mice in the conscious state was measured by using an indirect tail cuff method after administration of the saline, or [VO(tpps)] in saline, for 18 days. The measurement was carried out at room temperature (25 °C) because the instrument does not require prewarming of the animals. Five readings were obtained from each mouse, and the average was calculated.

Determination of Total Vanadium Concentration in the STZ Mice Treated with [VO(tpps)]. STZ mice were orally administered [VO(tpps)] in saline with doses of 10 mg of V/kg of body mass for the first 2 days, 4 mg of V/kg of body mass for the following 2 days, 10 mg of V/kg of body mass for the following 3 days, 7 mg of V/kg of body mass for the following 8 days, and then 10 mg of V/kg of body mass for the following 3 days. After administration of [VO(tpps)] in saline for 18 days, the mice were sacrificed under ether anesthesia and organs, such as the liver, spleen, pancreas, kidneys, lungs, heart, brain, fatty pads, muscles, and bones, were removed and lyophilized. The vanadium concentration was determined by neutron activation analysis at the Research Reactor Institute of Kyoto University by using a peak area corresponding to 1434.1 keV based on the $^{51}V(n,\gamma)^{52}V$ reaction (half-life of ^{52}V, 3.75 min).[26]

All animal experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and performed according to the guidelines for animal experimentation of KPU.

Statistical Analysis. Experimental results are expressed as the mean values ± standard deviations (SD). Statistical analysis was performed by using analysis of variance (ANOVA) or Student's *t*-test at the 5% ($p < 0.05$), 1% ($p < 0.01$), or 0.1% ($p < 0.001$) level of significance.

Results and Discussion

Preparation and Characterization of [VO(tpps)]. [VO(tpps)] was prepared according to the method used by Adler et al.[24] with slight modifications as described earlier, and [VO(tmpyp)] was prepared as described previously.[22] [VO(tpps)] was characterized by elemental analysis, visible absorption, IR, ESR, and mass spectra. The physicochemical parameters of [VO(tpps)], along with [VO(tmpyp)], are summarized in Table 1. From the calculated and found values in the elemental analyses (Table 1), DMF (2 moles) remained in the [VO(tpps)] sample because of its high boiling point (150 °C). In the visible absorption spectra of [VO(tpps)] dissolved in H_2O, a Soret band at 436 nm and two visible bands at 564 and 604 nm were observed. The apparent molar absorptivity of [VO(tpps)] was estimated to be 219.5×10^3, 18.8×10^3, and 7.3×10^3 M^{-1} cm^{-1} at 436, 564, and 604 nm, respectively, which are similar to the reported values.[25] In the IR spectra, the band due to the V=O stretching vibration was found to be at 1005 cm^{-1} for [VO(tpps)] and at 1007 cm^{-1} for [VO(tmpyp)], similar to the reported value at 1005 cm^{-1} for [VO(tpp)] (H_2tpp = tetraphenylporphyrin).[31] The FAB(−) mass spectra of [VO(tpps)] had a molecular ion peak (m/z) at 998, which supported the elemental analysis data (Table 1). The ESR spectra of [VO(tpps)] in H_2O were measured at room and liquid nitrogen temperatures and showed eight-line hyperfine splitting patterns at room temperature due to the unpaired electron of the ^{51}V nucleus ($I = 7/2$), indicating that only one mononuclear vanadium(IV) species is present in the examined sample solution. However, the ESR spectrum at liquid nitrogen temperature was relatively broad. The estimated ESR parameters (g and A values) are listed in Table 1, and the values indicate that the vanadium(IV) ion has a $VO(N_4)$ coordination sphere, based on the reference values of [VO(tmpyp)].[22] The small discrepancy in the ESR parameters for [VO(tpps)] and [VO(tmpyp)] (Table 1) might be due to a slight aggregation of [VO(tpps)] at liquid nitrogen temperature, which was observed in the ESR spectrum.

Stability of Oxovanadium(IV)–Porphyrin Complexes in the Serum Solution. The stability of oxovanadium(IV)–porphyrin complexes was studied in 4% bovine serum albumin and in the fresh blood serum of rats at 37 °C for 48 h. The fresh blood serum was collected from normal Wistar rats and centrifuged at 3000 rpm for 10 min. The oxovanadium(IV) state of porphyrin complexes was monitored by ESR. The ESR spectra of [VO(tmpyp)] in 4% bovine serum albumin as well as in the fresh blood serum of rats at room temperature had eight lines (data not shown), and those at 77 K (Figs. 2a and 2b) indicated the formation of more than one species, probably due to a slight interaction between [VO(tmpyp)] and the serum components. The spectrum of [VO(tpps)] were anisotropic at both

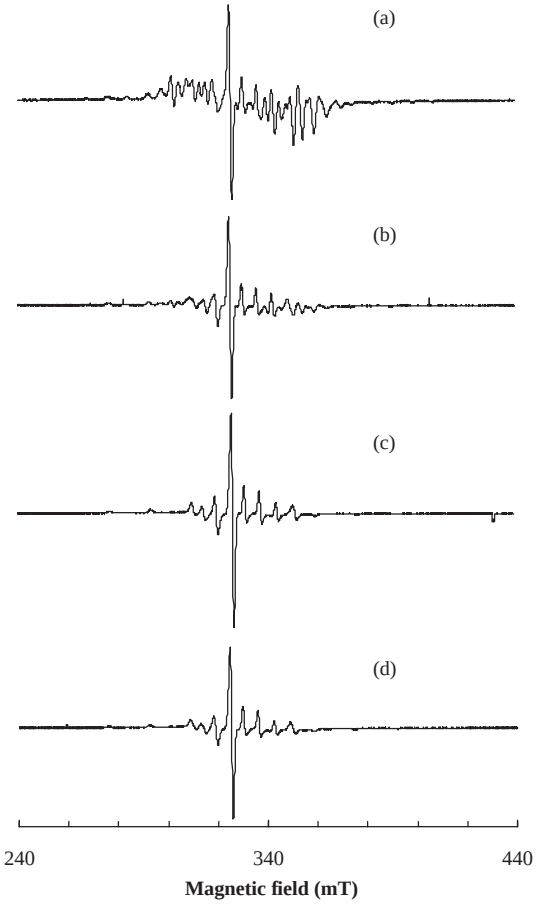

Fig. 2. ESR spectra at liquid nitrogen temperature (LNT) of [VO(tmpyp)] (1 mM) in 4% bovine serum albumin solution (a) and in the fresh rat blood serum (b) and [VO(tpps)] (1 mM) in 4% bovine serum albumin solution (c) and in the fresh rat blood serum (d) without ascorbate.

room (data not shown) and liquid nitrogen (Figs. 2c and 2d) temperatures. This indicates that there is a strong interaction between the sulfonate groups in [VO(tpps)] and the positive charge on bovine serum albumin[32] and fresh blood serum of rats. Kadish et al.[25] also observed similar phenomena, in which the addition of either 5% neutral surfactant Triton X-100 (TX-100), or 0.05 M cationic surfactant cetyltrimethylammonium bromide (CTAB), to [VO(tpps)] solutions induced anisotropy in the spectrum at room temperature. The ESR signal intensities due to the oxovanadium(IV) species of [VO(tpps)] and [VO(tmpyp)] were fairly stable in 4% bovine serum albumin as well as in the fresh blood serum of rats without ascorbate for 48 h (Fig. S1), whereas vanadium(IV) oxide sulfate was oxidized within 6 h, but stabilized in part by ascorbate.[22] However, the ESR signal intensity due to the oxovanadium(IV) species of [VO(tmpyp)] gradually increased with time up to 24 h in the fresh blood serum of rats (Fig. S1b). It might be due to the endogenous reduction of partially oxidized [VO(tmpyp)].

In Vitro Insulin-Mimetic Activity of Oxovanadium(IV)–Porphyrin Complexes. The in vitro insulin-mimetic activity of the complexes was examined based on both the inhibition of free fatty acid release and the enhancement of glucose uptake in isolated rat adipocytes treated with epinephrine in the

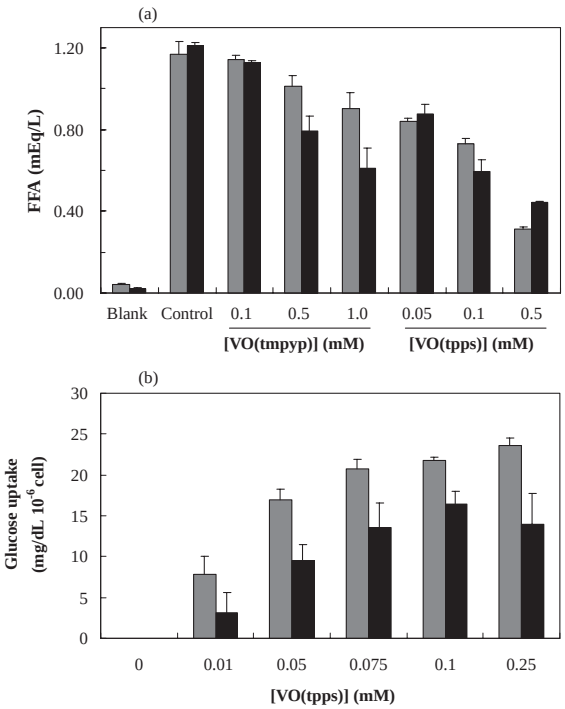

Fig. 3. Inhibitory effects of [VO(tmpyp)] (0.1–1.0 mM) and [VO(tpps)] (0.05–0.5 mM) on free fatty acid release (a) and enhancing effect of [VO(tpps)] (0.01–0.25 mM) on glucose uptake ability (b) in rat isolated adipocytes (2.5×10^6 cells mL^{-1}) treated with 0.01 mM epinephrine in the presence of 1 mg mL^{-1} glucose without (all dashed bars) and with sodium ascorbate (1 mM; all solid bars).

absence as well as in the presence of ascorbate.[26,27] The concentration-dependent inhibitory effects of [VO(tmpyp)] and [VO(tpps)] on free fatty acid release in isolated rat adipocytes treated with epinephrine were observed in the absence and in the presence of ascorbate (Fig. 3a). The apparent IC_{50} values in the absence and presence of ascorbate were estimated to be 28.70 ± 1.22 and 1.22 ± 0.59 mM for [VO(tmpyp)], 0.16 ± 0.01 and 0.16 ± 0.01 mM for [VO(tpps)] (Table 2), and 1.00 ± 0.34 and 0.34 ± 0.15 mM for vanadium(IV) oxide sulfate, respectively.[22]

[VO(tpps)] produced a concentration-dependent increase in glucose uptake in epinephrine-treated isolated rat adipocytes in the absence and presence of ascorbate (Fig. 3b); however, [VO(tmpyp)] did not enhance the glucose uptake ability in both cases. The apparent EC_{50} values of [VO(tpps)] were 0.03 ± 0.01 and 0.05 ± 0.01 mM in the absence and presence of ascorbate, respectively (Table 2). This suggests that ascorbate has no effect on both the IC_{50} and EC_{50} values of [VO(tpps)]. However, the significantly lower IC_{50} of [VO(tmpyp)] and vanadium(IV) oxide sulfate with sodium ascorbate strongly indicated that [VO(tmpyp)] and vanadium(IV) oxide sulfate were oxidized during incubation, and the oxovanadium(IV) state sustained by ascorbate is the active form that exhibits insulin-mimetic activity. As well, [VO(tpps)] has a higher insulin-mimetic activity than that of [VO(tmpyp)] or vanadium(IV) oxide sulfate. Moreover, the $E_{1/2}$ values for the reduction of [M(tmpyp)] (M = metal) are shifted positively by up to 1.2 V compared to $E_{1/2}$ values for the reduction of [M(tpp)]

Table 2. IC_{50} and EC_{50} Values for Oxovanadium(IV)–Porphyrin Complexes in Isolated Rat Adipocytes Treated with Epinephrine

Complex	In the absence of ascorbate		In the presence of ascorbate	
	Free fatty acid assay	Glucose-uptake assay	Free fatty acid assay	Glucose-uptake assay
	IC_{50}/mM	EC_{50}/mM	IC_{50}/mM	EC_{50}/mM
[VO(tpps)]	0.16 ± 0.01[a)]	0.03 ± 0.01	0.16 ± 0.01	0.05 ± 0.01
[VO(tmpyp)]	28.70 ± 1.22	not detected	1.22 ± 0.59	not detected

a) $p < 0.001$ vs [VO(tmpyp)] in the absence of ascorbate.

under the same solution conditions. At the same time, the values of $E_{1/2}$ for the reduction of [M(tpps)] differs from the $E_{1/2}$ of the corresponding [M(tpp)] species by only ± 0.05 V.[33,34] The $E_{1/2}$ value for the first reduction of [VO(tmpyp)] was -0.52 V and that of -0.98 V for [VO(tpps)] in DMF containing 0.1 M tetra-*n*-butylammonium perchlorate versus a saturated calomel electrode (SCE),[33,35] which suggests that the oxidation potential of [VO(tmpyp)] is lower than that of [VO(tpps)] under the same experimental conditions.

Metallokinetic Features of Oxovanadium(IV)–Porphyrin Complexes in the Blood of Healthy Rats as Estimated by Blood Circulation Monitoring-ESR. To understand the bioavailability of oxovanadium(IV)–porphyrin complexes, the metallokinetic features of paramagnetic oxovanadium(IV) species in the blood of healthy rats that received [VO(tmpyp)], [VO(tpps)], or vanadium(IV) oxide sulfate in the absence of ascorbate were analyzed using the blood circulation monitoring-ESR method.[28–30] A typical time-dependent change in a blood circulation monitoring-ESR spectra of [VO(tpps)] is shown in Fig. 4a; the central peak intensity of ESR spectra decreased slowly with time. The time courses of the oxovanadium(IV) species were estimated using the circulating blood of rats that were administered oxovanadium(IV)–porphyrin complexes, and the simulated curves which were fitted to the mean data on the basis of the one-compartment model using nonlinear least-squares regression are shown in Fig. 4b.[28–30] Both oxovanadium(IV) complexes decayed exponentially, and the clearance curves were fitted to the one-compartment model. The disappearance of the in vivo oxovanadium(IV) species in the circulating rat blood was time-dependent, indicating that the oxovanadium(IV) species absorbed into the blood were distributed to the peripheral tissues. In other words, the oxovanadium(IV) species are distributed in the short- and long-stay tissues, and then accumulated there, or they were redistributed in the bone, liver and kidney. Oxovanadium(IV) concentrations in the blood of rats that were given [VO(tpps)] remained significantly higher and longer ($t_{1/2} = 14.5 \pm 0.4$ min) than those in rats given [VO(tmpyp)] ($t_{1/2} = 5.4 \pm 0.6$ min; Fig. 4b) and vanadium(IV) oxide sulfate ($t_{1/2} = 3.6 \pm 0.6$ min).[30] The metallokinetic parameters of the oxovanadium(IV) species after the administration of [VO(tmpyp)], [VO(tpps)], and vanadium(IV) oxide sulfate are summarized in Table 3. The *AUC* and *MRT* values for the rats treated with [VO(tpps)] (*AUC*: 3.09 ± 0.18 μmol min mL^{-1}; *MRT*: 20.9 ± 0.6 min) were significantly higher than for those given [VO(tmpyp)] (*AUC*: 0.70 ± 0.10 μmol min mL^{-1}; *MRT*: 7.8 ± 0.8 min) and vanadium(IV) oxide sulfate (*AUC*: 0.27 ± 0.04 μmol min mL^{-1}; *MRT*: 5.2 ± 0.8 min). The values are related to the clearance

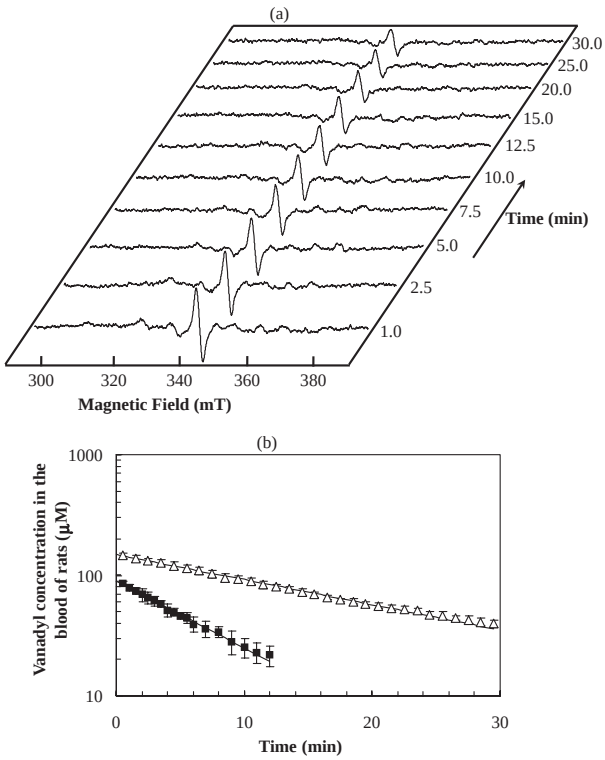

Fig. 4. In vivo blood circulation monitoring-ESR feature of oxovanadium(IV) species. A rat was administered [VO(tmpyp)] or [VO(tpps)] intravenously at a dose of 0.5 mg of V/kg body mass under anesthesia. Typical time-dependent blood circulation monitoring-ESR spectral change of [VO(tpps)] (a). ESR spectra were recorded at room temperature every 30 s following administration of the complex. Time courses of oxovanadium(IV) concentration in the blood of rats treated intravenously with [VO(tmpyp)] (■; $n = 3$), and [VO(tpps)] (△; $n = 3$) were monitored by the blood circulation monitoring-ESR method (b). The corresponding theoretical curves (solid lines) were fitted to the mean values. Complexes were dissolved in saline.

rates of the oxovanadium(IV) species from the blood of rats. In fact, the values of CL_{tot} and V_d in the rats treated with [VO(tpps)] (CL_{tot}: 3.2 ± 0.2 mL min^{-1} kg^{-1}; V_d: 66 ± 4 mL kg^{-1}) were significantly lower than those due to [VO(tmpyp)] (CL_{tot}: 14.1 ± 2.1 mL min^{-1} kg^{-1}; V_d: 109 ± 5 mL kg^{-1}) and vanadium(IV) oxide sulfate (CL_{tot}: 37.0 ± 5.5 mL min^{-1} kg^{-1}; V_d: 193 ± 12 mL kg^{-1}). These results suggested that the bioavailability of [VO(tpps)] was considerably higher than that of

Table 3. Metallokinetic Parameters of Oxovanadium(IV)–Porphyrin Complexes and Vanadium(IV) Oxide Sulfate[a)]

Metallokinetic parameter	Complex		
	[VO(tpps)]	[VO(tmpyp)]	Vanadium(IV) oxide sulfate
AUC/μmol min mL^{-1}	3.09 ± 0.18[b)]	0.70 ± 0.10	0.27 ± 0.04
MRT/min	20.9 ± 0.6[b)]	7.8 ± 0.8	5.2 ± 0.8
CL_{tot}/mL min^{-1} kg^{-1}	3.2 ± 0.2[b)]	14.1 ± 2.1	37.0 ± 5.5
V_d/mL kg^{-1}	66 ± 4[b)]	109 ± 5	193 ± 12
k_e/min^{-1}	0.048 ± 0.001[b)]	0.129 ± 0.013	0.191 ± 0.027
$t_{1/2}$/min	14.5 ± 0.4[b)]	5.4 ± 0.6	3.6 ± 0.6

a) Data are expressed as the means ± SDs for 3 rats. Rats were treated with vanadyl porphyrin complexes [VO(tpps)] and [VO(tmpyp)] at a dose of 0.5 mg V/kg body mass by *i.v.* injection under anesthesia. b) Significantly different at the 1% level of ANOVA in comparison with [VO(tmpyp)] or vanadium(IV) oxide sulfate.

[VO(tmpyp)] or vanadium(IV) oxide sulfate. Accordingly, [VO(tpps)] was anticipated to normalize the elevated blood glucose levels in the STZ-induced diabetic mice.

Effects of Oxovanadium(IV)–Porphyrin Complexes on Elevated Blood Glucose Levels and Oral Glucose Tolerance Ability in the STZ Mice. Based on the results of in vitro insulin-mimetic activity and metallokinetic features of oxovanadium(IV)–porphyrin complexes and vanadium(IV) oxide sulfate, the antidiabetic activity of [VO(tmpyp)], [VO(tpps)], and vanadium(IV) oxide sulfate was evaluated in the STZ mice. The changes in blood glucose levels in the STZ mice were evaluated after a single oral administration of the oxovanadium(IV)–porphyrin complexes at a dose of 15 mg of V/kg of body mass without ascorbate (Fig. S2). [VO(tpps)] significantly reduced the elevated blood glucose level within 8 h after the administration; the effect was sustained for at least 60 h. These results can be explained by the high bioavailability of [VO(tpps)] because it remained in the blood of rats for a long time in the absence of ascorbate based on the metallokinetic parameters in Table 3. On the other hand, the hypoglycemic activity of [VO(tmpyp)] was not confirmed at the same dose of [VO(tpps)] in the absence of ascorbate (Fig. S2). However, a single *i.p.* injection of [VO(tmpyp)] in saline at a dose of 5.0 mg V/kg body mass exhibited a hypoglycemic effect for 2–3 h after administration to the STZ rats, suggesting that the complex may be excreted quickly.[22] The ligand H_2tpps alone did not reduce the elevated plasma glucose levels in the STZ mice when compared with the saline-treated STZ mice (Fig. S2). The amount of H_2tpps administered to the STZ mice was equivalent to that of [VO(tpps)]. Moreover, the effect of vanadium(IV) oxide sulfate (15 mg V/kg body mass) was rather low in reducing the high blood glucose being remained at high levels around 400 mg dL^{-1} for 24 h (data not shown). The blood glucose levels in STZ-mice after 24 h following the single oral administration of saline alone and vanadium(IV) oxide sulfate in saline were almost unchanged. Woo et al.[21] reported that [VO(metf)$_2$] showed significant hypoglycemic activity in STZ rats after a single oral gavage at a dose of 0.6 mmol (30.6 mg) V/kg in 3% gum arabic. This complex reduced the elevated blood glucose level within 12 h after the administration; however, the effect was sustained for only 24 h. The blood glucose levels in STZ rats after 24 h following the single oral administration of 3% gum arabic alone and VO(metf)$_2$ were unchanged. This could be attributed to the fact that [VO(tpps)] showed both hydrophobic[34] and strong ionic[32] interactions with the blood serum components of mice as observed in interactions of bovine serum albumin or fresh blood serum of rats with [VO(tpps)]. In other words, there is a strong interaction with target components in glucose and lipid metabolites in the organs. On the other hand, [VO(tmpyp)] showed very low, or no, interaction with bovine serum albumin or fresh blood serum of rats, similar to neutral surfactant TX-100.[34] It was also reported that the tetra-anionic porphyrin complex is more hydrophobic than the tetra-cationic porphyrin complex.[34] As a result, [VO(tpps)] remained in the blood of mice for a long time and showed a significant sustainable hypoglycemic effect compared to [VO(tmpyp)], vanadium(IV) oxide sulfate and [VO(metf)$_2$]. Moreover, the effect of [VO(metf)$_2$] on the blood glucose levels was not significantly lower at any of the time points measured (compared with the zero time point) following treatment by acute *i.p.* injection at a dose of 0.12 mmol (6.11 mg) V/kg in 3% gum arabic. Similar results were obtained following treatment by acute *i.p.* injection of [VO(tpps)] in STZ mice and vanadium(IV) oxide sulfate in STZ rats[22] at a dose of 5.0 mg V/kg in saline. There was no significant difference between the blood glucose levels in saline- and [VO(tpps)]-treated STZ mice (data not shown), and vanadium(IV) oxide sulfate-treated STZ rats[22] at any of the time points measured. Hence, the present results indicated that [VO(tpps)], following acute oral gavage, has the highest hypoglycemic activity among [VO(tmpyp)], vanadium(IV) oxide sulfate, and [VO(metf)$_2$].

Since [VO(tpps)] was found to be a potent insulin-mimetic complex in vitro, as well as in vivo, evaluation based on the results of a single oral gavage and a single *i.p.* injection of vanadium(IV) oxide sulfate and oxovanadium(IV)–porphyrin complexes, we examined the effects of this complex on the change in the blood glucose levels in the STZ mice by daily oral administrations of [VO(tpps)] for 18 days. Figure 5a shows the change in the blood glucose levels in the STZ mice when saline alone or [VO(tpps)] in saline at doses in the range of 4–10 mg of V/kg of body mass was administered orally. When [VO(tpps)] was administered at 10 mg of V/kg of body mass, the blood glucose levels significantly decreased after 2 days. Subsequently, the doses of the complex were adjusted depending on the daily changes in the blood glucose levels. By such adjustments, the blood glucose levels were significantly decreased compared to those of the saline-treated STZ

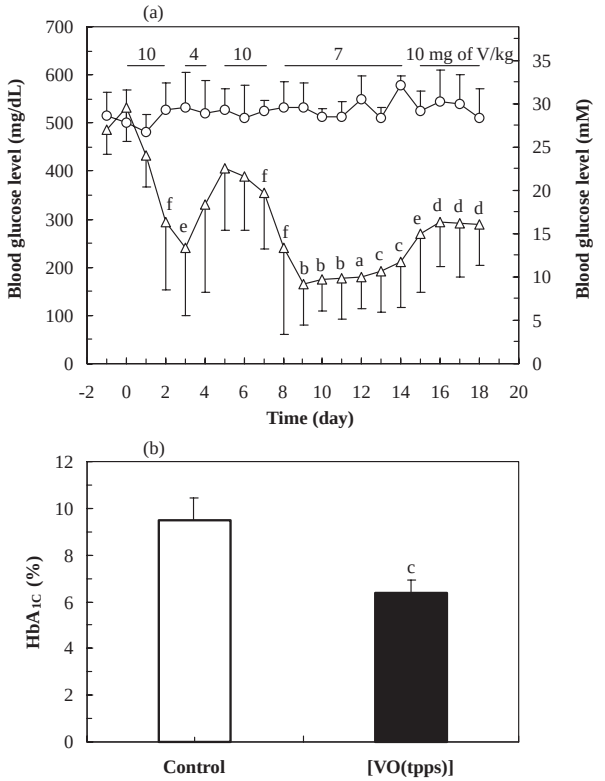

Fig. 5. Changes in blood glucose levels (a) in STZ mice after daily oral administration of saline or [VO(tpps)] in saline at the doses of 4–10 mg V/kg body mass for 18 days and amount of HbA_{1c} (b) after 18 days of administration ($n = 5$). Saline-treated STZ mice (○); STZ mice given [VO(tpps)] (4–10 mg of V/kg body mass) (△). Significance; a: $p < 0.00005$; b: $p < 0.0005$; c: $p < 0.001$; d: $p < 0.005$; e: $p < 0.01$; f: $p < 0.05$ vs saline-treated STZ mice ($n = 5$).

mice; the blood glucose levels remained below 200–300 mg dL^{-1} (11.1–16.7 mM) for 18 days, which was close to the blood glucose levels (100–200 mg dL^{-1} or 5.6–11.1 mM) in normal non-diabetic std: ddY mice. Previously, we found that daily *i.p.* injections of [VO(tmpyp)] (2.0 mg V/kg in saline) exhibited a hypoglycemic effect (around 300 mg dL^{-1} or 16.7 mM) from the 13th day when sodium ascorbate (20 mg/kg) was administered simultaneously; this indicated a synergicistic effect between [VO(tmpyp)] and sodium ascorbate.[22] During the present study, administration of [VO(tpps)] was accompanied by a significant loss of body mass associated with significantly reduced diet and water intake in all of the [VO(tpps)]-treated STZ mice as shown in Fig. S3.

We also examined the change in HbA_{1c} levels in the STZ mice treated with only saline, or [VO(tpps)] in saline, after 18 days administration (Fig. 5b). The rate of formation of HbA_{1c} is directly proportional to the ambient glucose concentration. Because erythrocytes are freely permeable to glucose, the level of HbA_{1c} in a blood sample provides a glycemic history of the previous 120 days for human, i.e., the average erythrocyte lifespan. HbA_{1c} testing first became available to clinical laboratories in the late 1970s.[36] Since then, the change in HbA_{1c} levels has been used as an index of glycemic control

Table 4. Serum Parameters and Blood Pressure in STZ Mice after Treatment with Saline Alone or [VO(tpps)] in Saline by Oral Administration for 18 Days

	Sample	
	Saline	[VO(tpps)]
Dose (mg V/kg)		4–10
n	5	5
Urea nitrogen/mg dL^{-1}	29.7 ± 10.7	16.3 ± 2.9[a)]
Glutamic oxaloacetic transaminase/U L^{-1}	75 ± 9	62 ± 20
Glutamic pyruvic transaminase/U L^{-1}	43 ± 9	23 ± 8
Triglycerides/mg dL^{-1}	103 ± 25	167 ± 29[a)]
Total cholesterol/mg dL^{-1}	190 ± 52	151 ± 32
Free fatty acid/mEq L^{-1}	1.458 ± 0.074	1.306 ± 0.145
Insulin/ng mL^{-1}	4.38 ± 0.23	4.50 ± 0.26
Blood pressure (mm of Hg)	137 ± 5.9	113 ± 17[a)]

a) $p < 0.05$ vs STZ mice given saline.

for both research and diabetic patients.[37] The HbA_{1c} level ($6.4 \pm 0.5\%$) in the [VO(tpps)]-treated STZ mice decreased significantly ($p < 0.001$) compared to that ($9.9 \pm 0.5\%$) of the saline-treated STZ mice, indicating a significant improvement of diabetic state in STZ mice treated with [VO(tpps)].

In order to determine whether [VO(tpps)] improved the glucose tolerance ability in the STZ mice, an oral glucose tolerance test was performed after treatment with [VO(tpps)] for 18 days. As shown in Fig. S4, after the oral administration of glucose at a dose of 1 g/kg body mass, the blood glucose levels of the saline-treated STZ mice increased to a maximum concentration of 490 mg dL^{-1} (27 mM) after 30 min, and then decreased gradually. However, the glucose level remained high. On the other hand, the elevation in the blood glucose levels (approximately 310 mg dL^{-1} or 17 mM) of the STZ mice treated with the [VO(tpps)] for 18 days were significantly suppressed than those of the saline-treated STZ mice. In other words, [VO(tpps)] exhibited a potent antidiabetic activity in the type 1 model STZ mice.

The levels of urea nitrogen, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, triglycerides, total cholesterol, insulin, and free fatty acid in the serum of the STZ mice after the treatment with only saline and with [VO(tpps)] in saline for 18 days are summarized in Table 4. The serum urea nitrogen level (16.3 ± 2.9 mg dL^{-1}), which indicates the degree of renal disturbance, of the [VO(tpps)]-treated STZ mice decreased significantly ($p < 0.05$) compared to the saline-treated STZ mice (29.7 ± 10.7 mg dL^{-1}). The glutamic oxaloacetic transaminase and glutamic pyruvic transaminase levels, which show the degree of liver disturbance, were suppressed compared to those of the saline-treated STZ mice. Total serum cholesterol levels (151 ± 32 mg dL^{-1}) after [VO(tpps)] treatment were lower as compared to that of the control STZ mice (190 ± 52 mg dL^{-1}), indicating that cholesterol metabolism improved due to the treatment with the oxovanadium(IV)–porphyrin complex. However, the serum triglycerides (167 ± 29 mg dL^{-1}) of the [VO(tpps)]-treated STZ mice significantly ($p < 0.05$) increased as compared to that of the control STZ mice (103 ± 25 mg dL^{-1}). The insulin levels of the [VO-

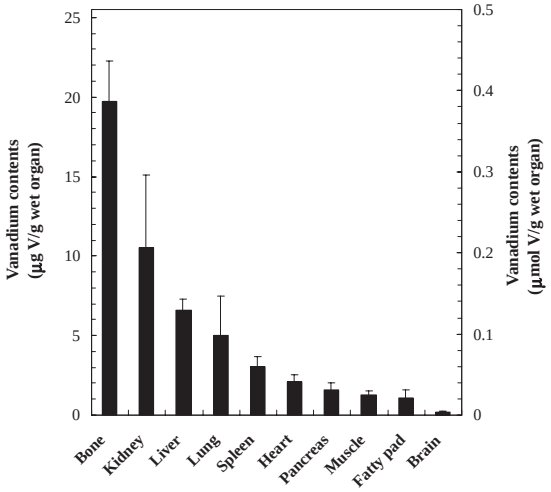

Fig. 6. Organ distribution of total vanadium in STZ mice after daily oral administration of [VO(tpps)] in saline at the doses of 4–10 mg V/kg of body mass for 18 days. After 18 days of administration, the mice were sacrificed under anesthesia with ether, and the organs were removed and lyophilized. Data are expressed as the means ± SDs for five mice.

(tpps)]-treated STZ mice (4.5 ± 0.3 ng/mL) were almost the same as those of the control STZ mice (4.4 ± 0.2 ng/mL). However, the free fatty acid level (1.31 ± 0.15 mEq L^{-1}) of the former group was slightly lower than that of the latter group (1.46 ± 0.07 mEq L^{-1}).

The systolic blood pressure (113 ± 17 mm of Hg) was significantly ($p < 0.05$) lower upon the administration of [VO(tpps)] when compared with that of the saline-treated STZ mice (137 ± 6 mm of Hg). Based on these results, it was suggested that [VO(tpps)] has a low, or no, hepatic and renal toxicity and improves cholesterol metabolism and blood pressure in STZ mice.

The total vanadium distribution in the STZ mice after treatment with [VO(tpps)] for 18 days was estimated using neutron activation analysis, which is the most reliable method for vanadium determination.[26] Vanadium was found to have accumulated in almost all of the tissues from the mice that were administered [VO(tpps)]. Vanadium was particularly found in decreasing levels in the bones, kidney, liver, lung, spleen, heart, pancreas, muscle, fatty pad, and brain (Fig. 6). It was reported that the vanadium levels in the tissues of rats treated with the bis(maltolato)oxovanadium(IV) complex were highest in the bone, intermediate in the kidneys and liver and low in the muscles and fat.[38] In these experiments, the rats were chronically fed bis(maltolato)oxovanadium(IV) in drinking water for 25 weeks, and the organs were removed at the termination of this chronic feeding study. Our results obtained from neutron activation analysis are in close agreement with those reported previously for bis(maltolato)oxovanadium(IV).[38] In contrast, in the STZ rats that were administered [VO(tmpyp)] and sodium ascorbate simultaneously, vanadium was detected at significantly higher levels in the liver and kidneys, followed by the spleen, bones, and pancreas.[22] This suggests that [VO(tpps)] has lower renal toxicity than [VO(tmpyp)] during the administration period.

On the basis of these results, [VO(tpps)] is the first example of an orally active oxovanadium(IV)–porphyrin complex with a $VO(N_4)$ vanadium coordination sphere that does not require the simultaneous administration of sodium ascorbate for treating type 1 diabetic mellitus animals. However, daily *i.p.* injections of [VO(tmpyp)] were effective in the STZ rats only when they were simultaneously administered with sodium ascorbate.[22]

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Supporting Information

Figures S1–S4 are in PDF format. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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