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Studies on the Usefulness of Radioisotopes in Pharmaceutical Sciences

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The use of radioisotopes and radiation is essential in the research field of life science. This article describes the studies on the usefulness of radioisotopes in pharmaceutical sciences, especially focusing on the results of our research group as follows. (i) Mixed disulfide formation catalyzed by Cu(II) in relation to the radioprotective ability of a radioprotector, cysteamine (2-mercaptoethylamine). (ii) Suppression of reactive oxygen species (ROS) in the skin of live animals given oral administrations of Zn(II) and its complexes in relation to the skin damage under ultra-violet (UV) light. (iii) Improvement of diabetes mellitus by insulin-mimetic vanadyl(IV) (VO(II)) complexes.

1. Introduction

The use of radioisotopes and radiation is indispensable in the research of life science, especially in pharmaceutical sciences. Research topics in pharmaceutical sciences are mainly classified into three research fields as follows.

(1) Trace elements in living systems and environment, where many kinds of tracers and neutron activation analysis are used.
(2) Radioisotopes in the development of new pharmaceutics, where a wide variety of radioisotopes are needed for radioimmunoassay, radioreceptor assay, pharmacokinetics, imaging by autoradiography, radioprobes, drug delivery systems, and gene technology.
(3) Radiopharmaceutics in nuclear medicine, where not only in vitro and in vivo diagnostic radiopharmaceutics but also in vivo therapeutic radiopharmaceutics have extensively been developed.

Since 1971, we have used many tracers, and performed neutron activation analysis (NAA) method (Table 1) to know the chemical action mechanism of radioprotective agents, uptake of trace elements and glucose into the cells and organs, state of essential trace elements in organs of animals, and metal incorporations in subcellular particles and organs in experimental animals that were treated with metallo-pharmaceutics as well as metal complexes as candidates for future metallo-pharmaceutics.

This review article describes progress in our investigation on the research of pharmaceutical sciences, focusing on the following three topics.

(i) Mixed disulfide formation catalyzed by Cu(II) in relation to the radioprotective ability of a radioprotector, cysteamine (2-mercaptoethylamine).
(ii) Suppression of reactive oxygen species (ROS) in the skin of live animals given oral administrations of Zn(II) and its complexes in relation to the skin damage under ultra-violet (UV) light.
(iii) Improvement of diabetes mellitus by insulin-mimetic vanadyl(IV) (VO(II)) complexes.

2. Mixed Disulfide Formation Catalyzed by Cu(II) in Relation to the Radioprotective Ability of Cysteamine (2-mercaptoethylamine)

The concept of chemical protection against radiation damage in mammal was established in 1949–1955.[1,2] A wide variet-

TABLE 1: Tracers and radio-nuclides used in our research group

Tracers

^{35}S-cysteamine, ^{35}S-cysteine		(1971)
^{3}H-glucose		(1990)
^{54}Mn		(1990)
2-deoxy-D-[1-^{3}H]glucose		(1999)
^{48}V	(AVF cyclotron)	(2003)
^{65}Zn	(AVF cyclotron)	(2005)

Multi-tracers (RIKEN ring cyclotron)

^{7}Be, ^{22}Na, ^{47}Ca, ^{46}Sc, ^{48}V, ^{51}Cr, ^{52}Mn, ^{59}Fe, ^{58}Co, ^{65}Zn, ^{67}Ga, ^{74}As, ^{75}Se, ^{84}Rb, ^{85}Sr, ^{87}Y, ^{88}Zr, ^{89}Zr, ^{99m}Tc, ^{96}Tc, ^{97}Ru, ^{103}Ru, and ^{101m}Rh (2001)

Neutron activation analyses

^{55}Mn(n, γ)^{56}Mn	846.6 keV	(1985)
^{51}V(n, γ)^{52}V	1434.1 keV	(1987)
^{198}Pt(n, $\gamma\beta^{-}$)^{199}Au	158 keV	(1988)
^{76}Se(n, γ)^{77m}Se	162 keV	(1988)
^{197}Au(n, γ)^{198}Au	411.8 keV	(1988)
^{127}I(n, γ)^{128}I	443.7 keV	(1990)
^{65}Cu(n, γ)^{66}Cu	1039.2 keV	(1992)
^{58}Fe(n, γ)^{59}Fe	192.3 keV	(1992)
^{64}Zn(n, γ)^{65}Zn	1115.5 keV	(1992)
^{27}Al(n, γ)^{28}Al	1778.9 keV	(1996)
^{39}K(n, γ)^{40}K	1460.8 keV	(1997)
^{133}Cs(n, γ)^{134}Cs	795.8 keV	(1997)
^{31}P(n, α)^{28}Al	1778.9 keV	(2004)

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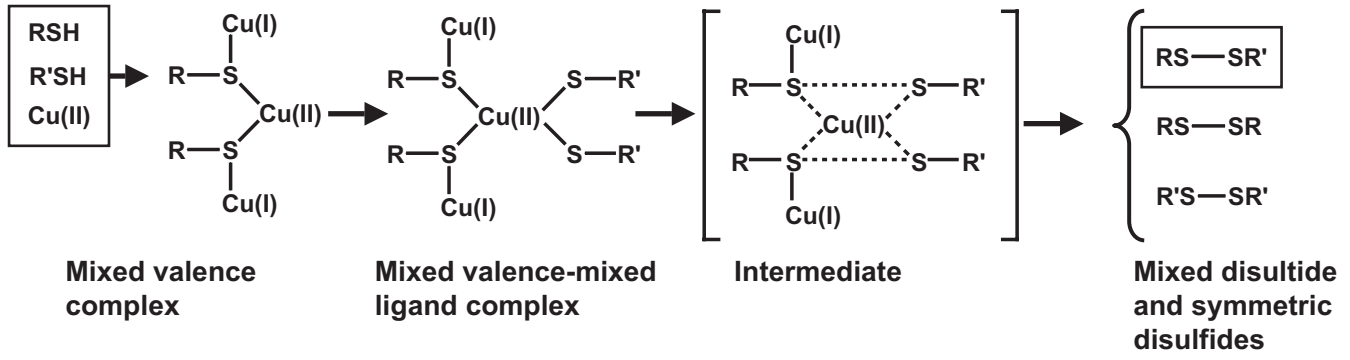


Figure 1. Possible mechanism for mixed disulfide formation through mixed valence-mixed ligand complex catalyzed by Cu(II).

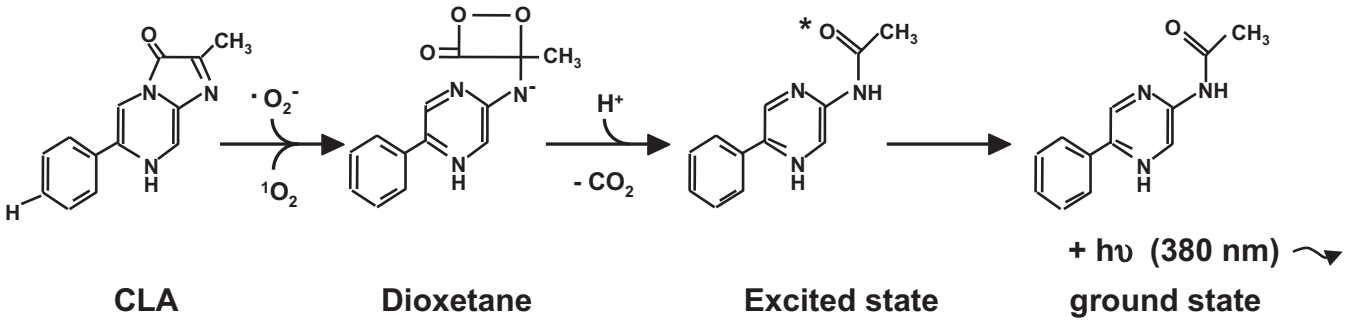


Figure 2. CLA (2-methyl-6-phenyl-3,7-dihydroimidazo-[1,2-a]pyrazin-3-one) and its reaction with superoxide anion ($\cdot O_2^-$) or singlet oxygen ($^1O_2$).

ies of radioprotectors involving cysteamine were proposed by many researches. Together with the development of radioprotectors, the action mechanism for the chemical protection has intensively been studied. The main hypotheses for the chemical protection are summarized as follows.[3]

1) Reactions of radioprotectors with free radical species that are formed during the reactions of radiation and water as solvent in cells or organs and biomolecules such as proteins, lipids and nucleic acids.
2) Energy transfer of radiation through biomolecules to radioprotectors.
3) De-oxygenation effect by reductive radioprotectors.
4) Mixed disulfide formation between sulfur-containing radioprotectors such as cysteine or cysteamine (2-mercaptoethylamine) and thiol groups of proteins or enzymes, by which such proteins and enzymes are protected against radiation damage.
5) Chelate theory, which proposes the chelate formation of radioprotectors with metal ions such as Cu and Fe that in turn catalyze the oxidative damage in cells or organs under exposure of radiation.

Among these hypotheses, we have been interested in the mixed disulfide formations consisting of different types of thiol compounds, to which trace amount of metal ions may contribute. In order to know the contribution of complex formation of a radioprotector, cysteamine (Cyst), which is a well-known agent, we examined the effects of Cu(II) on the formation of the mixed disulfide in the system of Cyst and cysteine, glutathione, or bovine serum albumin by using ^{35}S-Cyst or ^{14}C-cycteine at pH 7.4. Separation of the mixed disulfides were achieved by electrophoresis, and the amounts of the mixed disulfides were estimated by measuring the radioactivity. Remarkable catalytic effects were observed, in which the order of the formation yield (%) of mixed disulfides were as follows: 70% for Cyst-cysteine system, >53% for Cyst-glutathione system, and >36% for Cyst-albumin system.[4]

For these reactions, we speculated the mechanism following the visible absorption spectrometry (Figure 1). When Cyst is mixed with Cu(II), a mixed valance complex, which contains Cu(I) and Cu(II) states, is formed.[5] When another type of thiol compound is added, this compound binds to the vacant coordination sphere of central Cu(II) and a mixed valence mixed ligand complex will be formed. This complex is altered to as an intermediate, in which two sulfur atoms from different thiol compounds interact each other and a new mixed disulfide will be formed together with formations of each symmetric disulfide compound.

3. Suppression of Reactive Oxygen Species (ROS) in the Skin of Live Animals Given Oral Administrations of Zn(II) and Its Complexes in Relation to the Skin Damage under Ultra-violet (UV) Lights

When the skin is exposed to ultra-violet (UV) light, the reactive oxygen species (ROS) involving superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot$OH), singlet oxygen ($^1O_2$), and lipidperoxides or their radicals (LOOH or LOO$\cdot$) have been proposed to form in the skin, which in turn induce skin aging, phototoxicity, inflammation, and malignant tumors. Such proposal depended on the results obtained from the separated most outer layer of the skin (epidermis) or homogenated tissues. Therefore, contradictory results were often reported, probably due to the lack of experimental data in live animals. Actually, there are a few reports on in vivo detection of ROS generation in the skin.[6,7]

In 2000, we reported the in vivo real-time detection and two-dimensional imaging of the generated ROS in the skin of a live animal after UVA light (320−400 nm) exposure, by means of a chemiluminescent (CL) probe (CLA) (Figure 2) combined with a new ultralow-light imaging apparatus equipped with a CCD camera (NightOWL). The method was proposed to be very useful not only in characterizing the ROS generated in the UV light-exposed skin but also in searching for the protective or preventive compounds against the UV light-induced skin injury or disorder.[8,9]

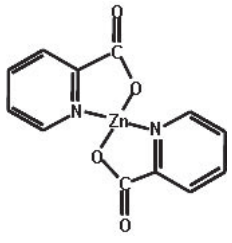
Because a significant difference was observed between intrinsic and UVA-induced CL in the skin of live mice, the sorts of ROS were identified by using topical applications of

typical and specific ROS scavengers. Applications of superoxide dismutase (SOD) or β-carotene were found to greatly reduce UVA-induced CL in the skin. Furthermore, SOD also highly reduced intrinsic skin CL, while β-carotene hardly reduced it. The facts that SOD is a potent $^1O_2$ quencher (quenching rate constant = 2.6×10^9 $M^{-1}s^{-1}$) as well as an $\cdot O_2^-$ scavenger (second order rate constant = 2.0×10^9 $M^{-1}s^{-1}$) and β-carotene is a typical $^1O_2$ quencher (quenching rate constant = $3–30 \times 10^9$ $M^{-1}s^{-1}$) suggest that the intrinsic CL was due mostly to $\cdot O_2^-$ and that the UVA-induced CL was due predominantly to $^1O_2$ in the mouse skin. We thus demonstrated the direct in vivo evidence for the intrinsic occurrence of $\cdot O_2^-$ and the UVA-induced formation of $^1O_2$ intrinsically generated in the skin of live mice.

In searching for the protective compounds against UVA light, we use both organic and inorganic compounds or complexes. Among many inorganic elements, Zn has been used as an anti-inflammatory agent for many years. Then we have used Zn of both ionic and complex forms. When $ZnCl_2$ was topically applied to the live hairless mice, the CL intensity due to ROS was significantly suppressed. Based on the finding of this new physiological role of Zn, $ZnCl_2$ was intraperitoneally injected to the hairless mice, and examined whether administered Zn(II) protects the skin again UVA light-induced damage or not. Zn affect was observed after three days of daily $ZnCl_2$ administration for 3 consecutive days, however, the effect disappeared after seven days. The study was further intended to examine whether orally administered $ZnCl_2$ protects against UVA-light induced skin damage in term of suppression of ROS generation or not.

Orally administered $ZnCl_2$ suppressed ROS generation in the skin of mice under UVA exposure even at three days after the last Zn administration.

Encouraged with the results, a Zn complex, bis(picolinato)Zn(II) [$Zn(pa)_2$], with the partition coefficient of 0.018 in n-octanol-saline system at 37 °C was orally administered in hairless mice. $Zn(pa)_2$ exhibited a long-term protective effect again UVA-induced ROS generation rather than the effect of $ZnCl_2$.[10,11]



$Zn(pa)_2$

The reason why orally administered Zn(II) or its complex exhibits antioxidative activity in the skin under UVA exposure is difficult to explain, where many factors contribute. We examined the induction of metallothionein (MT), which is proposed to have an antioxidative activity in several organs involving the skin of animals, in HaCaT cells treated with $ZnCl_2$, and found that MT levels in the cells increased when compared with the non-treated cells.

In addition, to know the mechanism for the different features of protective ability of $ZnCl_2$ and $Zn(pa)_2$, we used ^{65}Zn, which was provided from RIKEN (Wako, Japan) in the form of $^{65}ZnCl_2$. $^{65}Zn(pa)_2$ was prepared from $^{65}ZnCl_2$.

The time-dependent distribution of ^{65}Zn in the skin of hairless mice treated with $^{65}ZnCl_2$ or $Zn(pa)_2$ by single oral administration at a dose of 10 mg Zn per kg of body weight was observed (Figure 3). Interestingly, ^{65}Zn levels in the skin of mice treated with $^{65}Zn(pa)_2$ were significantly higher than those of mice treated with $^{65}ZnCl_2$ at 12, 24, and 72 h after administration, indicating that the actions of Zn on the skin are more enhanced and prolonged by the supplementation of

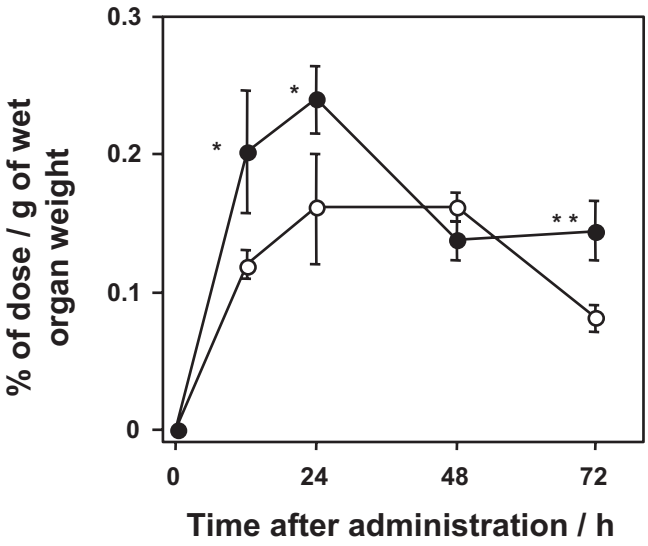


Figure 3. Time-dependent distribution of ^{65}Zn in the skin of hairless mice after single oral administration of ^{65}Zn-labeled $ZnCl_2$ (open circles) or $Zn(pa)_2$ (closed circles). Significance levels: * $p < 0.05$ and ** $p < 0.01$ of $Zn(pa)_2$ group vs. $ZnCl_2$ group at the same sampling time.

$Zn(pa)_2$ than those of $ZnCl_2$.

4. Improvement of Diabetes Mellitus by Insulin-mimetic Vanadyl(IV) (VO(II)) Complexes

Among several lifestyle-related diseases involving hypertension, hyperlipemia, obesity, diabetes mellitus, myocardial infarction, and cerebral embolism, the diabetes mellitus (DM) is one of the most important diseases, since the patients suffering from DM in the 21st century increase more than in the 20th century and they develop many secondary complications such as atherosclerosis, microangiopathy, renal disfunction and failure, cardiac abnormality, diabetic retinopathy, and ocular disorders. In general, DM is classified into type 1 DM caused by destruction of pancreatic B cells, and type 2 DM caused by aging, obesity, spiritual stress or other environmental factors, that are treated by daily injections of insulin and several types of synthetic therapeutic compounds, respectively, for survival. Unfortunately, the injections of insulin several times in a day are painful and give the patient stress, especially in baby and young people less than 25 years old, and synthetic compounds often exhibit some severe side effects containing hypoglycemia, anemia, hepatic disorder, and agranulocytosis. To overcome these defects, the creation of new classes of the therapeutic compounds based on new concepts is urgently anticipated.

As one of the new concepts, we examined the possibility to use metal ions and their complexes on oral administrations in place of insulin injection, in the first stage of our study, and found the usefulness of biometals such vanadyl (VO(II)) and zinc (Zn(II)) as insulin-mimetic metal ions, in which the inhibition of the release of free fatty acids (FFA) from the isolated rat adipocytes (adipose cells prepared from the eqididymal fat tissue) by metal ions was evaluated.

In fact, when streptozocin-induced type 1-like DM rats (STZ-rats) were given daily ip injections of $VOSO_4$, their serum glucose levels dropped from hyperglycemic to normal levels within 2 days and serum free fatty acids (FFA) levels also dropped to normal. However, the plasma insulin levels remained low, suggesting that the VO(II) action is peripheral. Total V was found to be incorporated into most organs as well as adipose tissues, as determined by neutron activation analysis (NAA). V was incorporated into the organs in the following order (μg V g^{-1} wet weight): kidney > liver > bone > pancreas;

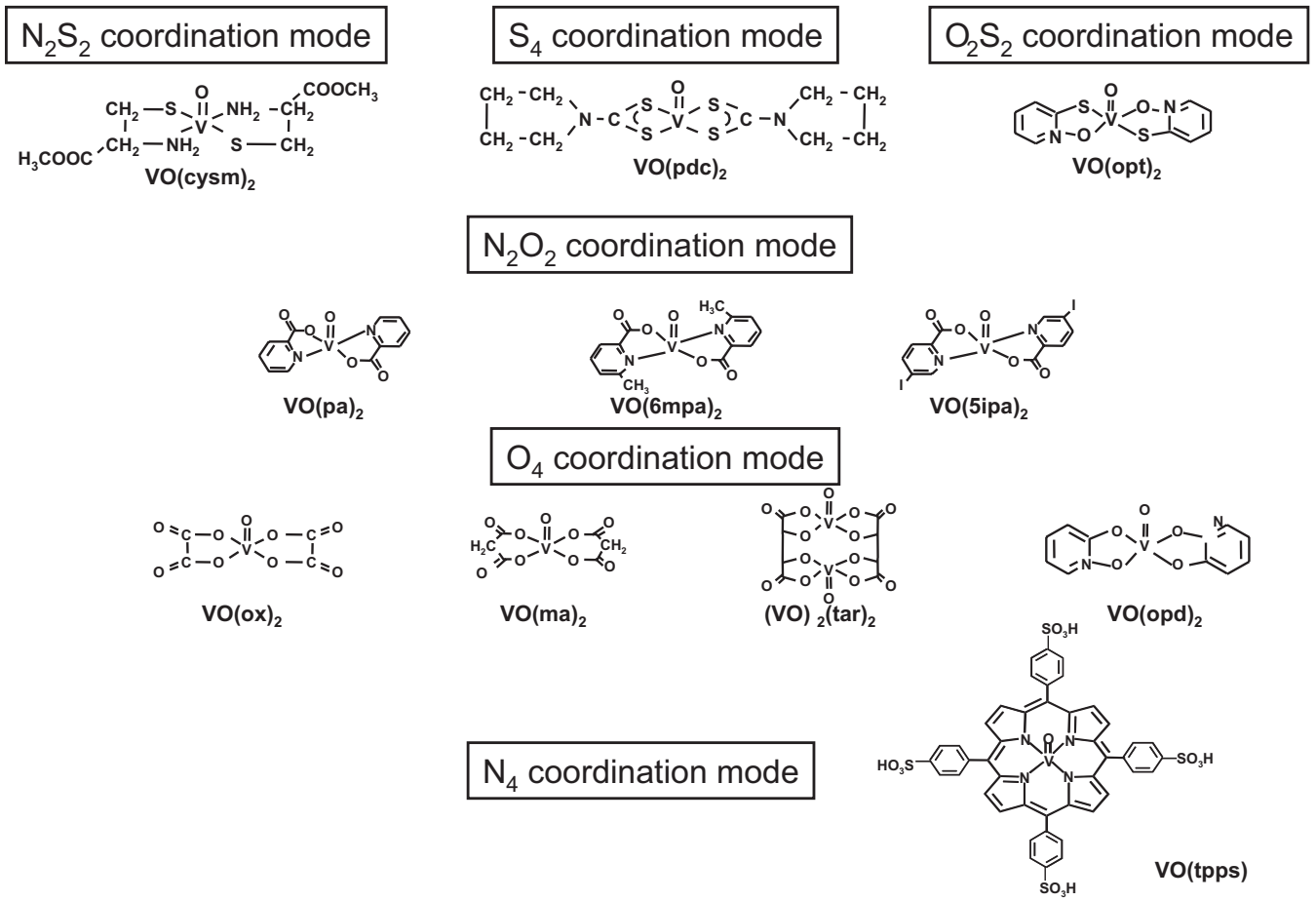


Figure 4. Insulin-mimetic vanadyl (VO(II)) complexes with different coordination modes.

it was also incorporated into the supernatant of the kidney and mitochondria of the liver. V was thus assumed to act in part on the islet of the pancreas, on mineralization of bone, on electron transport systems or induction of metallothionein (MT) in the kidney, and on the liver.[12]

It is interesting to note that no significant differences in V uptake of normal and STZ-rats was observed. Both VO(II) and total V in several organs of STZ-rats treated with $VOSO_4$ could be determined by electron spin resonance (ESR) spectrometry at 77 K. In almost all organs, approximately 90% of the V was found to be present in the VO(II) form.

Possible ligands to the VO(II) in organs were also estimated by ESR spectrometry for freshly isolated organs such as liver, kidney, and serum of STZ-rats treated with $VOSO_4$, in which a characteristic eight-line signal due to VO(II) was observed. To know the coordination mode around the detected VO(II) species, ESR parameters ($A_\parallel$-value as a hyperfine coupling constant and $g_\parallel$-factor as a universal constant, characteristic of the paramagnetic species) were compared with those of several model VO(II) complexes with various coordination modes around the VO(II). The relationship between two ESR parameters ($A_\parallel$ vs. $g_\parallel$) for VO(II) complexes suggested that the VO(II) species in tissues was predominantly in an oxovanadium (VO(II)) form with a square pyramidal structure, in which VO(II) was coordinated with four oxygen ligands of either water or oxyamino acid residues in proteins.[12]

Because uptake of V in animals given oral $VOSO_4$ is very low, we tested some VO(II) complexes to enhance the bioavailability of $VOSO_4$ in STZ-rats.

In 1990, we first found that bis(methylcysteinato) [$VO(cysm)_2$]-, bis(oxalato) [$VO(ox)_2$]-, bis(malonato) [$VO(mal)_2$]-, and bis(salicylaldehydo) [$VO(sal)_2$]-oxovanadium(IV) as well as bis((+)-tartarato) [$(VO)_2(tar)_2$]-dioxovanadium(IV) complexes with either the $VO(N_2S_2)$ or $VO(O_4)$ coordination mode show the normoglycemic effects in STZ-rats when orally administered daily, the order being $VO(mal)_2 > VO(cysm)_2 > (VO)_2(tar)_2 > VO(sal)_2 > VO(ox)_2$ with the action of dose-dependency in the V concentration range of 1–10 mg kg^{-1} body weight of rats.[13] This finding indicated that the chelation of VO(II) enhances the bioavailability of VO(II) and the change of ligand around the VO(II) alters highly the insulin-mimetic action.

In addition, ESEEM (electron spin echo envelope modulation) spectrometry was applied to reveal a more detailed in vivo coordination structure of the VO(II) state in the organs of rats treated with $VOSO_4$. The ESEEM spectra of the kidney and liver measured at 77 K demonstrated the occurrence of nitrogen(N)-coordination to a certain percentage of VO(II), when they were compared with several model VO(II) complexes and proteins. The ratios of N coordinating VO(II) were estimated as 70–80% in the liver, and 50–55% in the kidney. Isotopic portions of the ^{14}N hyperfine coupling were estimated as $|A_{iso}| = \sim 5.0$ MHz for the liver, and ~5.2 MHz for the kidney, indicating that the coordinating N was an amino nitrogen. Thus, in vivo coordination of Lys ε-amino or N-terminal α-amine of a protein (or a peptide) to VO(II) was suggested.[14]

In finding of insulin-mimetic VO(II) complexes in vitro, we evaluate both the enhancing effect of glucose uptake and the inhibitory effect of free fatty acids (FFA) release in the isolated rat adipocytes treated with epinephrine (adrenaline). FFA and glucose levels in the outer solution of the cells were determined. In view of the importance of finding more active VO(II) complexes, we prepared a large number of complexes with different coordination modes and found the therapeutic candidates as shown in Figure 4.[15–18]

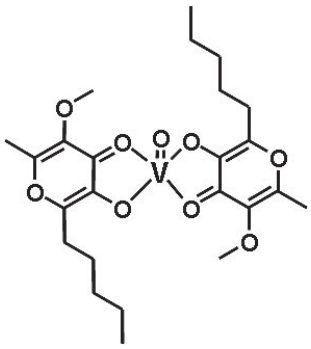
Among them, bis(picolinato)oxovanadium(IV)[$VO(pa)_2$] complex with the $VO(N_2O_2)$ coordination mode is particularly interesting in having advantage for examining the structure-

activity relationship by adding suitable substituents on the picolinate as a leading ligand.[19]

On the basis of the results, it was concluded that in vitro insulin-mimetic activity, real-time metallokinetic character and in vivo antidiabetic action of $VO(pa)_2$ complexes are closely related to their chemical structures; the position of a substituent changes the sensitivity of the complex in animals.[20] The long-term activity of orally administered $VO(6mpa)_2$ in STZ-rats was observed, which might be related to the accumulation of V in organs of animals.

Organ distribution of total V in STZ-rats receiving daily ip treatments of $VO(6mpa)_2$ (59 μmol V kg^{-1} body weight for the first day and 39 for 2 days, and then 20 for 11 days, and then no treatment for 7 days) was then examined by NAA method. The order of V concentration ($\mu g\ g^{-1}$ wet weight) was as follows: bone > kidney > spleen > liver > adipose > pancreas > lung > heart > brain, suggesting that accumulation of V in the bone accounts the long-term acting of $VO(6mpa)_2$.[19] In addition, the formation of ternary complexes consisting of 6-methylpicolinate-VO(II)-protein or amino acids was revealed by ESEEM, indicating also the long-term acting of $VO(6mpa)_2$.[21]

To find another types of insulin-mimetic complexes, we focused on bis(3-hydroxy-4-pyronato)oxovanadium(IV)compl ex with $VO(O_4)$ coordination mode, since this complex has also an advantage to examine the structure-activity relationship. During the study, we found an excellent VO(II) complex with allixin, which was isolated from dried garlic (Allium Sativum L.) as a new non-sulfur phytoalexin.



$VO(alx)_2$

Bis(allixinato)oxovanadium(IV) [$VO(alx)_2$] exhibited not only a high in vitro insulin-mimetic activity in terms of FFA release inhibitory and glucose-uptake enhancing activities in isolated rat adipocytes but also a high hypoglycemic effect in STZ-mice by both ip injections and oral administrations.[22]

V, which was determined by NAA method, was detectable in almost all tissues of STZ-mice given $VO(alx)_2$, particularly, bone, spleen, liver, pancreas, and skeletal muscle, in this order, similarly to those given bis(maltolato)oxovanadium(IV) [$VO(ma)_2$], which has the same coordination structure to $VO(alx)_2$ (Table 2). Interestingly, $VO(alx)_2$ showed a higher distribution of V in the muscle than $VO(ma)_2$. These results suggested that $VO(alx)_2$ has a potent hypoglycemic effect due to the higher distribution of V to the muscle, which is an important target tissue for insulin, than that of $VO(ma)_2$.[22]

Based on the results, we further examined whether or not $VO(alx)_2$ treats type 2 DM with low insulin sensitivity. Oral treatment of obsesity-linked type 2 diabetic KK-A^y mice with $VO(alx)_2$ for 4 weeks normalized hyperglycemia, glucose intolerance, hyperinsulinemia, hyperleptinemia, hypercholesterolemia, and hypertension, however, it has no effect on hypoadiponectinemia. From these results, $VO(alx)_2$ was proposed to enhance not only insulin sensitivity but also leptin sensitivity, which in turn improve diabetes, obsesity, and hypertention in the animals.[23]

V concentration in the tissues of KK-A^y mice treated with $VO(alx)_2$ was determined by NAA method (Table 2). V was detectable in almost all organs; the order of distribution was as follows: bone > kidney in liver > spleen > adipose > pancreas > muscle.[23] Previously, we hypothesized that V accumulated in the bones may be due to the long-term acting characteristic of V compounds. Although V compounds were suggested to be toxic to the bone marrow and cause anaemia, no impairment was observed in the hepatic and renal systems of $VO(alx)_2$-treated KK-A^y mice. From these results, $VO(alx)_2$ was proposed to treat not only type 1 diabetic animals but also obesity-linked type 2 diabetes.

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TABLE 2: Organ distribution of vanadium in mice treated with $VO(alx)_2$ and $VO(ma)_2$

Animal	Administered complex	Vanadium content / $\mu g\ g^{-1}$ of wet tissue)							
		Plasma	Bone	Liver	Kidney	Spleen	Pancreas	Muscle	Adipose
Mice (ddY)			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
STZ-mice	$VO(ma)_2$ *		4.6	1.1	0.76	1.2	0.91	0.10	
	$VO(alx)_2$ *		4.0	1.0	0.87	1.1	0.56	0.16	
KK-A^y mice		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
KK-A^y mice	$VO(alx)_2$ **	1.7	31	5.3	5.2	4.0	1.1	0.8	1.1

* Administration doses of ip injections: 5 mg (98 μmol) V /kg/day at the first day and then it was decreased to 0.1 mg (2 μmol) V /kg/day for 13 days.
** Administration doses of oral administrations: 3–7 mg (59–137 μmol) V /kg/day for 4 weeks.

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