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# Membrane transport of vanadium compounds and the interaction with the erythrocyte membrane

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## Contents



## Abstract

In the present work, the membrane transport and the biotransformation of vanadate, bis(maltolato)oxovanadium ($VO(ma)_2$), and vanadyl acetylacetonate ($VO(acac)_2$) were investigated to explore the relationship with their insulin-like activity. Cellular uptake kinetics were performed by ICP-AES and EPR. The uptake of $VO(acac)_2$ and $VO(ma)_2$ by human erythrocytes showed intracellular vanadium level higher than $NaVO_3$ and the membrane transport of these two vanadyl complexes was presumed to be via the passive diffusion mechanism. A fraction of vanadyl was oxidized to anionic vanadium(V) species and also entered the cells by the anion

*Abbreviations:* DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulphonate; DPH, 1,6-diphenyl-1,3,5-hexatrience; EPR, electron paramagnetic resonance; HEPES, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid; $VO(ma)_2$, bis(maltolato)oxovanadium; $VO(acac)_2$, vanadyl acetylacetonate; IOV, inside-out vesicles; ROV, right-side out vesicles; ICP-AES, inductively coupled plasma atomic emission spectrometry; FT-IR, Fourier transformed infrared spectroscopy.

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channel. The stability of $VO(acac)_2$ and $VO(ma)_2$ to oxidation in human erythrocyte membrane vesicles was investigated using EPR. $VO(ma)_2$ was found to be more sensitive to oxidation than $VO(acac)_2$ in aqueous buffer solution. However, in the presence of membrane vesicles, the oxidation of $VO(ma)_2$ and $VO(acac)_2$ was retarded and the differences between them became insignificant. Thus, the lifetime of vanadium complexes might be prolonged in physiological fluids. The interaction with membranes appears to be important in the stabilization of vanadyl complexes. Meanwhile, structural changes of membrane proteins were also observed. The higher uptake of the vanadyl complexes and the observed changes of membrane proteins might attribute to their insulin-mimetic mechanisms and toxicities.


*Keywords:* Vanadium complexes; Transport; Biopotency; Diabetes

## 1. Introduction

In recent years, many vanadium compounds have been synthesized and screened for their insulin-mimetic activity. The structure–activity relationship and the mechanisms of these compounds have been explored [1,2]. Their insulin-mimic effects were perceived to be due to inhibition of protein tyrosine phosphatases, or the action on the insulin receptor or the targets distal to the receptor in the insulin signaling pathway [3–5], but the pharmacological mechanism is not well understood. The factors determining their insulin-like activity are still unclear. The difference in the therapeutic potency of various vanadium compounds cannot be simply interpreted on the basis of any proposed mechanism. In fact, the therapeutic efficacy is not only determined by a compound's activity, but also by the absorption, distribution, metabolism and elimination and toxicity (ADME/T) properties. The differences in these aspects are important factors, which could lead to the difference in pharmacological potency of vanadium compounds. The ADME/T is mostly determined by the stability, lability and redox chemistry of these compounds [6,7] and these properties are dependent in turn on the nature of ligands [8–10]. Under physiological conditions, both the +4 and +5 oxidation states of vanadium are accessible kinetically and thermodynamically [11–15]. So the shuttle between the two oxidation states, +4 and +5, may play a pivotal role in the biological effects of vanadium compounds. However, there are only limited data available on the redox stability and interconversion between oxidation states of vanadium in biological media. It has been demonstrated that $VO(acac)_2$ and $VO(ma)_2$ are superior to vanadate in their high activity and extremely low toxicity [10,16], but it cannot be simply explained by any mechanism. For this sake, the membrane transport of vanadate, $VO(acac)_2$ and $VO(ma)_2$ and the events occurring in interaction with the erythrocyte membrane were investigated and compared in order to pursue the factors which determine the biopotency of the vanadium compounds.

## 2. Materials and methods

### 2.1. Materials

Human red blood cells (Red Cross Blood Center, Beijing, China); 4,4′-diisothiocyano-2,2′-stilbene disulphonate (DIDS), *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 1,6-diphenyl-1,3,5-hexatrience (DPH) were from Sigma. All other chemicals were of analytical grade and all solutions were prepared in the double-distilled de-ionized water. Stock solutions of ammonium metavanadate, $VO(acac)_2$ and $VO(ma)_2$ were prepared in HEPES-buffered isotonic saline (Buffer H) (NaCl, 135 mM; HEPES, 15 mM; pH 6.8) just before use. All experiments were performed once in triplicate.

### 2.2. Preparation of human erythrocyte membranes

Erythrocyte ghosts, inside-out vesicles (IOV) and right-side out vesicles (ROV) were prepared as described below. The protein contents of the erythrocyte membranes were assayed by the Lowry method [17].

#### 2.2.1. Preparation of leaky erythrocyte ghosts

Human erythrocyte ghosts were prepared according to Dodge [18] with modifications. Packed human erythrocytes were washed three times with Buffer H by centrifuging at $2000 \times g$ for 10 min. The cells were lysed in 30 volumes of 5 mM HEPES (pH 8.0) (5H8) at 4 °C. The ghosts were subsequently concentrated by centrifuging at $15{,}000 \times g$ for 10 min and washed twice in the same buffer.

#### 2.2.2. Preparation of IOV

IOV were prepared according to literature [19]. One milliliter of unsealed erythrocyte membrane, suspended in 5H8 buffer, was diluted to 40 ml with 0.5 mM HEPES (pH 8.0) (0.5H8) and kept in ice for 0.5–1.5 h. Then the membrane suspension was centrifuged at $28{,}000 \times g$ for 30 min, then resuspended in 1 ml of 0.5H8 by vortex mixing and passage through a No. 27 gauge needle three to five times to accomplish vesiculation. The vesicle

suspension (2 ml) was overlaid on 3 ml of Dextran barrier (4.46 g of Dextran T-70 dissolved in 100 ml of 0.5H8, $d = 1.015$ mg ml$^{-1}$). After centrifugation for 40 min at 29,000 × $g$, the top band was collected and washed with 40 volumes of 0.5H8 buffer at 29,000 × $g$ for 30 min.

#### 2.2.3. Preparation of ROV

The ROV used were the intact ghosts prepared by incubating the leaky ghosts in 10 volumes of the isotonic HEPES solution at 37 °C for 40 min and packed by centrifugation at 15,000 × $g$ for 30 min.

The two types of the prepared vesicles, IOV and ROV, were identified using sidedness assays with acetylcholine esterase and glyceraldehyde-3-phosphate dehydrogenate [20]. The percentages of sidedness accessibility thus obtained for both IOV and ROV preparations are in agreement with those previous reported [20].

### 2.3. Membrane transport of vanadium compounds

The experiments were performed by the procedure described by Cantley et al. [13] as follows for intact erythrocytes. The washed packed erythrocytes were suspended in the same volume of Buffer H at a hematocrit of 50% (v/v). For the transport studies, only vanadium compounds were added to the suspensions. For the inhibition experiments, before the addition of vanadium compounds, DIDS was added and the mixture was incubated at 37 ± 1 °C for 30 min. The final solution contained 375 μM oxovanadium compound and the erythrocytes at a hematocrit of 10% (v/v) (containing $5 \times 10^8$ cells ml$^{-1}$), but also 125 μM DIDS in the inhibition experiments. The samples in microfuge tubes were incubated at 37 ± 1 °C. After 5, 10, 15, 30, 60 and 120 min, the tubes were placed in the ice-water bath for 2 min to stop the entry of vanadium and centrifuged at 2000 × $g$ immediately. The pellets were washed twice with chilly Buffer H, followed by mixing with deionized water, vortexed vigorously for 30 s and then left for 20 min at room temperature. After centrifugation at 15,000 × $g$ for 20 min, the supernatant, combined with that obtained from ensuing washings was used for the determination of cytosolic vanadium concentration $C_{in}$. The samples for $C_{in}$ analysis were digested with ultrapure nitric acid and perchloric acid. Vanadium was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Leeman Labs Inc., USA).

The concentration dependence of membrane transport was studied by the same method described above. The erythrocyte suspensions were incubated with the vanadium compounds with the concentration of oxovanadium compounds varied from 0 to 800 μM for 30 min.

### 2.4. EPR measurement

All EPR spectra were recorded on a Bruker EPR 300 X-band spectrometer with power 20 mw, 100 KHz modulation, amplitude 790 G, central magnetic field 3480 G and time constant 0.655 s. The spectrometer was operating at room temperature (20 ± 0.5 °C) in 1 mm outer diameter (o.d.) quartz tubes and was calibrated using a powdered sample of 2,2-bis(4-*tert*-octylphenyl)-1-picryhydrazyl (2.0037 g).

For kinetic studies, the EPR spectra were recorded at different time after mixing the $VO(acac)_2$ or $VO(ma)_2$ with a 50% red blood cell suspension. The total concentration of vanadium complexes was 1.0 mM.

For interaction of $VO(acac)_2$ and $VO(ma)_2$ with human erythrocyte ghosts, the samples were prepared by mixing the stock solution of $VO(acac)_2$ and $VO(ma)_2$ with the leaky ghost suspension, IOV and ROV suspension, respectively. The mixed suspensions were incubated at 37 ± 0.5 °C for 5, 60 and 120 min, respectively under aerobic conditions. The EPR spectra were scanned.

### 2.5. Erythrocyte osmotic fragility test

Stock solutions of ammonium metavanadate, $VO(acac)_2$ and $VO(ma)_2$ were prepared in physiological saline solutions. The fragility tests according to Dacie and Lewis [21] were carried out upon addition of 100 μl of these solutions to 1 ml of red blood cell suspension and incubated for 2 h at 37 °C. Fifty microliters of the preparation thus obtained was added to 5 ml of concentrated saline solutions ranging from 0.2 to 0.85% NaCl. The suspensions were centrifuged at 2000 × $g$ for 10 min and hemolysis was estimated from the absorbance at 540 nm of hemoglobin released into the supernatant on a Shimadzu UV210 UV–vis spectrophotometer. The results were expressed as percentage of total hemolysis by 0.1% NaCl solution.

### 2.6. Lipid fluidity of erythrocyte membranes

After incubating the intact erythrocyte ghosts with the oxovanadium compounds at 37 ± 1 °C for 30 min, the membranes were separated from the solution by centrifugation at 15,000 × $g$ for 20 min and resuspended in 2 ml of Buffer H. After the addition of 0.1 ml of DPH ($2 \times 10^{-6}$ M), the suspensions were left for 20 min at room temperature. The lipid fluidity was measured by the fluorescence polarization method [22] on a HITACHI F-4010 fluorescence spectrophotometer ($\lambda_{ex} = 360$ nm; $\lambda_{em} = 430$ nm; slit = 5 nm).

### 2.7. FT-IR studies on the conformational changes of membrane proteins

After incubating the intact erythrocyte ghosts with the oxovanadium compounds at 37 ± 1 °C for 30 min, the membrane was separated from the buffer by centrifugation at 15,000 × *g* for 20 min, resuspended in $D_2O$ and stored at 4 °C for 24 h for complete hydrogen–deuterium exchange. For routine FT-IR measurements, a suspension of 30 mg protein per ml membrane was used. All FT-IR spectra were measured on a Bio-Rad FTS-65A FT-IR spectrometer with a HgCdTe detector and water-cooled globar source. The spectra of the membrane protein fractions were obtained by subtracting the buffer absorbance from the recorded spectra by the reported procedure [23]. A set of water vapor spectra recorded under identical conditions was used to eliminate the absorbance of residual water vapor. The resolution of the spectra was enhanced by means of standard DIGILAB software [24]. The second-derivative spectra were calculated with optimal bandpass parameters between 0.4 and 0.8 to discern as many as component bands and to ensure a flat baseline between 1700 and 1600 $cm^{-1}$. Curve fitting was performed with the Bio-Rad Win-IR CURVEFIT program.

## 3. Results

### 3.1. Membrane transport

Representative results of studies on the cellular uptake kinetics for the various oxovanadium species are shown in Fig. 1. The differences in membrane transport of vanadate, $VO(acac)_2$ and $VO(ma)_2$ were clearly displayed. The intracellular level of vanadium increased quickly after exposure to $VO(acac)_2$ or $VO(ma)_2$, reaching a very high level ( ~ 14 μg) within 5 and 15 min, respectively and leveling off thereafter to a limiting value about 16 μg. For vanadate, on the other hand, the vanadium influx displayed a biphasic feature, which is in accordance with early studies [13]. The initial fast rising step is followed by a second slower step and the intracellular vanadium level increases slowly with time. The other informative results are the different effects of DIDS, the specific blocker of the anion channel, on the transport of vanadate and the other two complexes. The transport of vanadate was inhibited almost completely, but that of $VO(acac)_2$ or $VO(ma)_2$ was slightly affected. It is reasonable to postulate that the uncharged complexes, $VO(acac)_2$ and $VO(ma)_2$ diffuse in their intact forms through the membrane lipid bilayer via simple diffusion, while the vanadate anions diffuse into the cell via the anion channel, which is consistent with the earlier result [13]. The linearity of the dose-dependence curves given in Fig. 2 supports the passive diffusion model for these vanadium complexes. The rates of diffusion for $VO(acac)_2$ and $VO(ma)_2$ are nearly the same.

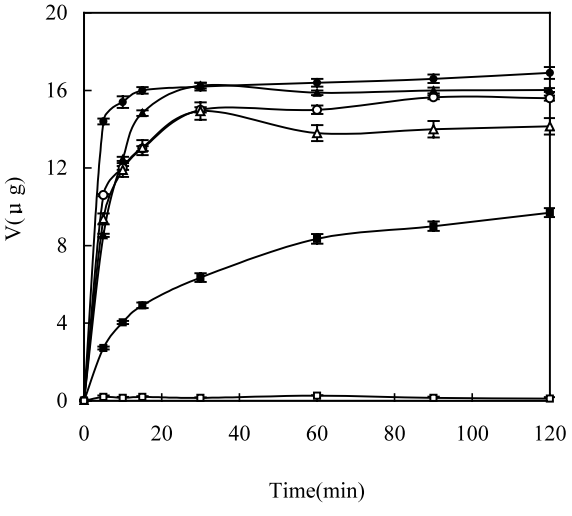


Fig. 1. Kinetic uptake and the DIDS inhibition of the uptake of various oxovanadium compounds (375 μM) by human erythrocytes. The *x*-axis represents the incubation time and the *y*-axis represents the level of vanadium in the cell ($C_{in}$) at different time. (●, $VO(acac)_2$; ○, $VO(acac)_2$+DIDS; ▲, $VO(ma)_2$; △, $VO(ma)_2$+DIDS; ■, $NaVO_3$, □, $NaVO_3$+DIDS). Data represent the means ± S.D. ($n = 3$).

Membrane transport of $VO(acac)_2$ and $VO(ma)_2$ was further studied using EPR spectroscopy. The spectra were recorded after different incubation times. The EPR spectra of $VO(acac)_2$ and $VO(ma)_2$ in Buffer H exhibit an eight-line isotropic feature of vanadyl due to the coupling of the single $d_{xy}$ electron with the nuclear spin of the vanadium atom ($I = 7/2$, $^{51}V = 99.8\%$ natural abundance) (Fig. 3(1)), corresponding to the extracellular environment. Within the cells, the medium becomes more viscous than the surrounding fluid outside

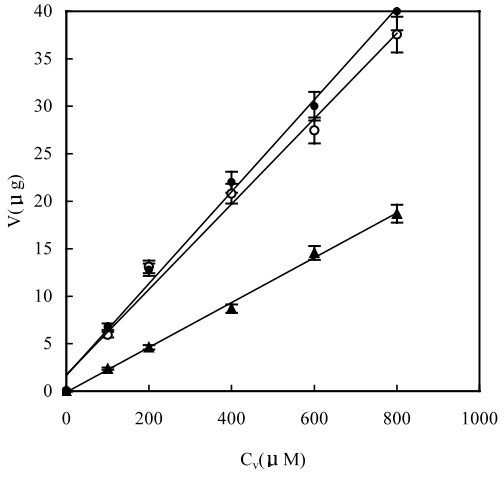


Fig. 2. Uptake curves as a function of concentration of various oxovanadium compounds. The dose is given on the *x*-axis as concentration of vanadium compound (●, $VO(acac)_2$; ○, $VO(ma)_2$; ▲, $NaVO_3$). Data represent the means ± S.D. ($n = 3$).

the cells and, in addition, the vanadyl ions could be bound to immobile cellular components. Thus the emergence of anisotropic EPR signals indicates the entry of vanadyl species. As shown in Fig. 3, the appearance of anisotropic EPR spectra became evident after 2 min incubation for $VO(acac)_2$, but 12 min for $VO(ma)_2$.

In summary, both results indicate that the diffusion rates of the $VO(acac)_2$ and $VO(ma)_2$ are more rapid than that of vanadate, while $VO(acac)_2$ is more rapid than $VO(ma)_2$. The data are consistent with the hypothesis that the difference in therapeutic efficacy between the vanadyl complexes and vanadate is due to different transport mechanisms.

Since the transport of two vanadyl complexes can be inhibited by DIDS to a small extent, it is assumed that the portions inhibited are due to the transformed species of these two oxovanadium compounds. That is to say, a fraction of the vanadyl complexes could possibly be oxidized to the negative charged oxidation products and enter the cells via the anion channel. Therefore, the oxidation of the two vanadium complexes in the simple buffer solution and the membrane suspensions were investigated using EPR spectroscopy to investigate the redox stability and interconversion between oxidation states of vanadium in biological media, which may affect bioavailbility.

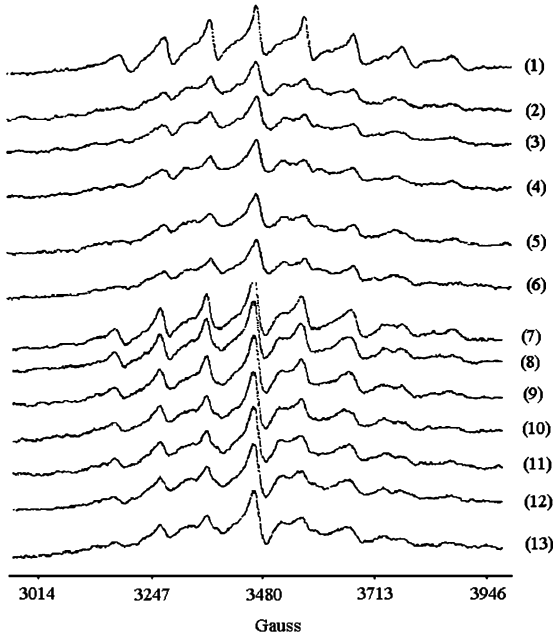


Fig. 3. EPR spectra of $VO(acac)_2$ and $VO(ma)_2$ at 20 °C after incubation with red blood cells. The fresh human red cells were prepared as described in Section 2 and incubated as a 50% (v/v) suspension in Buffer H with 1.0 mM $VO(acac)_2$ and $VO(ma)_2$ for different incubation time. (1) 1.0 mM $VO(acac)_2$ in Buffer H; (2)–(6): 1.0 mM $VO(acac)_2$ with human erythrocytes in Buffer H at 2.0; 4.0; 6.0; 9.0; 12.0 min, respectively. (7) 1.0 mM $VO(ma)_2$ in Buffer H; (8)–(13): 1.0 mM $VO(ma)_2$ with human erythrocytes in Buffer H at 2.0, 7.0, 9.0, 12.0, 15.0, 17.0 min, respectively.

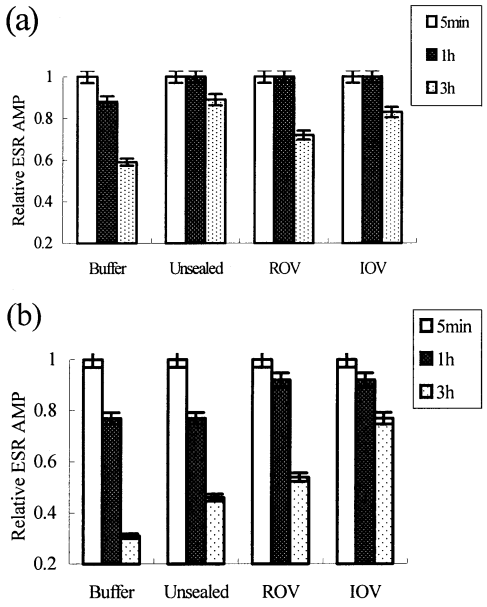


Fig. 4. Oxidative decay of the VO EPR signal: (a) $VO(acac)_2$; (b) $VO(ma)_2$ in buffer solution, unsealed, ROV and IOV membrane suspensions. The 1.0 mM $VO(acac)_2$ was added to different suspensions (at a membrane protein concentration of 3.4 mg $ml^{-1}$) and the EPR spectra at 20 °C were recorded after different incubation time at 37 °C. The $y$-axis represents the relative EPR amplitude ratio of the central portion of the EPR spectra of $VO(acac)_2$ and $VO(ma)_2$. Data represent the means ± S.D. ($n = 3$ or 4).

### 3.2. Oxidation of $VO(acac)_2$ and $VO(ma)_2$ in erythrocyte membrane suspensions

The results of the EPR studies reported here revealed the difference in redox stability of $VO(acac)_2$ and $VO(ma)_2$ in contact with membrane suspensions and in simple buffer solutions. The intensity reduction of the central signal of the EPR spectra was used to estimate the relative amounts of the two oxidation states [25]. The results given in Fig. 4 showed that in chemical systems, both $VO(acac)_2$ and $VO(ma)_2$ were susceptible to oxidation and $VO(ma)_2$ was more sensitive. In the presence of unsealed ghosts, as well as ROV and IOV, the rate and the extent of oxidation are suppressed but to different extent. It is reasonable to assume that the + 4 oxidation state is stabilized by binding to erythrocyte membrane constituents, mainly those at the inside of the erythrocyte membrane, because IOV retarded the oxidation to a greater extent than ROV.

The unsealed ghosts inhibited oxidation of $VO(acac)_2$ most, then the IOV and followed by ROV. For $VO(ma)_2$, the inhibition by IOV ranked first, followed by ROV and the unsealed membranes. As we know, one of the differences between IOV or ROV and unsealed membrane is that the former contains membrane barriers. $VO(ma)_2$ is more susceptible to air oxidation than $VO(acac)_2$ [16]. In unsealed membrane suspen-

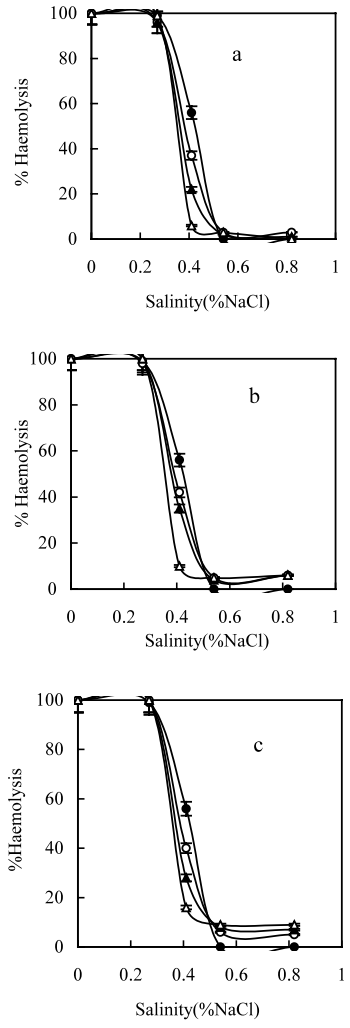


Fig. 5. Effect of: (a) $NaVO_3$; (b) $VO(acac)_2$; (c) $VO(ma)_2$ on osmotic fragility. % hemolysis is plotted vs. % NaCl (●, control; ○, 0.15; ▲, 0.30; △, 0.45 mM) Data represent the means ± S.D. ($n = 3$).

sions, its reaction with oxygen is kinetically favored. On the other hand, the oxidation of $VO(ma)_2$ by oxygen can be delayed in IOV and ROV, in which the membrane barriers can lower the oxygen partial pressure. The +4 oxidation state of $VO(ma)_2$ was thus stabilized by binding with the membrane constituents.

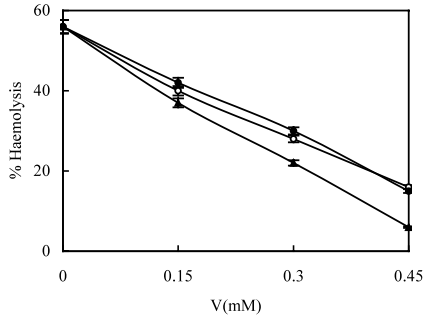


Fig. 6. Hemolysis vs. dose relationship for $VO(acac)_2$, $VO(ma)_2$, and $NaVO_3$. The percentage of hemolysis at fixed (0.41%) salinity is plotted against the vanadium concentration (●, $VO(acac)_2$; ○, $VO(ma)_2$; □, $NaVO_3$).

Based on these results, we can conclude that the V(IV) is subjected to oxidation under the physiological conditions. However, in the presence of erythrocyte membrane, the rate and extent of oxidation is reduced. The stability of $VO(acac)_2$ against oxidation is higher than $VO(ma)_2$. The vanadium(V) compounds formed by oxidation are most likely recognized and transported by the anion channel, as shown in Fig. 1.

### 3.3. Effects of vanadium compounds on erythrocyte osmotic fragility

To evaluate the effects of the oxovanadium compounds on the stability of the erythrocyte membrane, the percentage of hemolysis in the presence of the oxovanadium compounds was determined by measuring the osmotic fragility. The changes of salinity of 50% hemolysis (Fig. 5) showed that all three compounds decreased the fragility of the erythrocyte membranes, and the dose–response curves (as shown in Fig. 6) are all roughly linear.

### 3.4. Effects of vanadium compounds on the lipid fluidity of erythrocyte membrane

The incorporation of a foreign compound into the lipid bilayer may perturb the membrane structure and thus induce the changes in lipid fluidity, which indicates perturbation of the membrane structure. The lipid fluidity of erythrocyte membrane was assessed by DPH fluorescence polarization method. Based on the fluorescence spectra of DPH labeled erythrocyte membranes, the polarization as well as the microviscosity was calculated. The results shown in Table 1 indicated that all the three vanadium compounds increased the fluorescence polarization, indicating the increased order of the membrane structure and decreased fluidity. The hydrophobic species, $VO(acac)_2$ and $VO(ma)_2$, exerted stronger effect on the microviscosity, presumably due to stronger hydrophobic interaction with the lipid bilayer.

### 3.5. FT-IR studies on the conformational changes of membrane proteins

The FT-IR spectra showed that the secondary structure and the conformation of membrane proteins were sensitive to vanadium binding (Fig. 7). For the intact ghosts, the amide I band (1600–1700 $cm^{-1}$), which reflecting the secondary structure and conformation was resolved to 10 component peaks at 1608.4, 1616.8, 1626.9, 1636.0, 1644.5, 1652.7, 1660.3, 1667.9, 1675.5 and 1683.4 $cm^{-1}$. After reacting with $NaVO_3$, the peaks 1636 and 1652.7 $cm^{-1}$ shifted to 1632.2 and 1653.2 $cm^{-1}$, respectively, the peaks at 1608.4, 1616.8 and

Table 1
Effects of vanadium compounds on the lipid fluidity of DPH-labeled human erythrocyte membranes

| $C$ (mM) | Polarization | | | Microviscosity | | |
|---|---|---|---|---|---|---|
| | $NaVO_3$ | $VO(acac)_2$ | $VO(ma)_2$ | $NaVO_3$ | $VO(acac)_2$ | $VO(ma)_2$ |
| 1.0 | 0.224±0.002 | 0.237±0.002 | 0.237±0.002 | 1.90±0.02 | 2.13±0.02 | 2.13±0.02 |
| 0.1 | 0.223±0.002 | 0.230±0.002 | 0.232±0.002 | 1.88±0.02 | 2.00±0.02 | 2.04±0.02 |
| 0.0 | | 0.220±0.001 | | | 1.83±0.02 | |

Data represent the means±S.D. ($n = 3$); $P < 0.05$ vs. control.

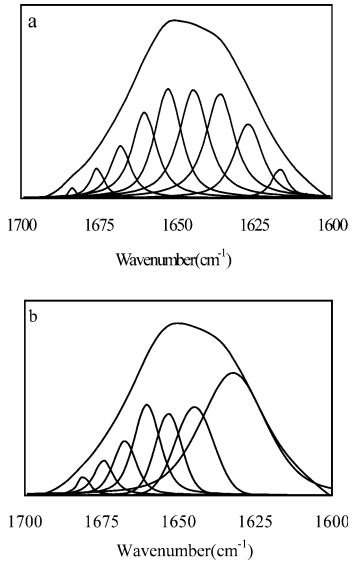


Fig. 7. The FT-IR fitted individual component bands of human erythrocyte membrane proteins spectra of amide I region (1700–1600 $cm^{-1}$) in the presence and absence of $NaVO_3$. (a) Erythrocyte membrane proteins; (b) incubation of erythrocyte membrane with $NaVO_3$.

Table 2
Quantitative estimation of secondary structure of human erythrocyte membrane proteins in the presence of various vanadium compounds (final concentration of vanadium compounds: 0.8 mM)

| | $\alpha$ | $\beta$ | $T$ | $\gamma$ |
|---|---|---|---|---|
| Control | 32.1±1.2 | 36.2±2.1 | 11.0±2.0 | 20.7±2.7 |
| $NaVO_3$ | 25.4±1.7 * | 49.6±1.9 * | 9.1±1.8 | 16.0±2.2 |
| $VO(acac)_2$ | 29.1±1.8 | 45.7±1.6 * | 8.9±1.3 | 16.2±2.3 |
| $VO(ma)_2$ | 31.9±1.6 | 43.2±2.2 * | 8.1±1.8 | 14.0±2.9 |

Data represent the means±S.D. ($n = 3$).
* $P < 0.05$ vs. control.

1626.9 $cm^{-1}$ disappeared. Based on FT-IR studies of proteins published recently [26], the main peaks at 1652–1660 $cm^{-1}$ are associated with α-helical structures. The bands appearing at 1626, 1634, 1636, 1675 $cm^{-1}$ can be assigned to β-sheet and the band at 1644, 1646 $cm^{-1}$ to random structures. Three other bands, which appear at 1667, 1680 and 1683 $cm^{-1}$, can be assigned as the turns. The weak band 1608 would be from absorbance of aromatic side-chain residues [27,28]. To assess the relative contribution of each type of secondary structure present, it is assumed that all of the component bands have the same absorption coefficient. Referring to Fig. 7, the percentages of each one component were calculated. Similar band-fittings were made for the membrane proteins in the presence of $VO(acac)_2$ and $VO(ma)_2$. The results listed in Table 2 indicate the increased β-sheet content.

## 4. Discussion

It has been reported that oral administration of $VO(acac)_2$ or $VO(ma)_2$ induced a faster and greater lowering of hyperglycemia than the simple salt, the effects persisted for up to 3 months without significant toxicity, and $VO(acac)_2$ was found more effective than $VO(ma)_2$ [10]. The present results indicate that the difference in uptake rate and the accumulation in cytosol might be a crucial factor determining the therapeutic efficacy. The uptake was studied by determining the temporal changes of cytosolic vanadium by ICP-AES and the time of appearance of vanadyl in the cells by EPR method. The former gives a picture of the whole process, while the EPR method provided a way to estimate the time consumed before the appearance of vanadyl in the cytosol. The results indicate that two vanadyl complexes entered the cell more rapidly and reached a higher vandium level inside the cells than vanadate. The difference in accumulation between vanadyl complexes and vanadate shown here may be due to the different transport mechanism. Our results showed that vanadate, as reported before [13], entered the cell through anion channel, while $VO(acac)_2$ and $VO(ma)_2$, both entered the cell mainly by simple passive diffusion in their intact forms. This difference is explainable on the basis of their zero charge and hydrophobicity. The transport of these two vanadyl complexes is further complicated by the oxidation of a fraction of vanadyl to V(V) anionic species. Results obtained by Caravan et al. [6] demonstrated that in aqueous solution, $VO(ma)_2$ is slowly oxidized by molecular oxygen to $[VO_2(ma)_2]^-$. The major species of

$VO(acac)_2$ in the NaCl containing solution is a hydrolytic species, a positive-charged 1:1 complex, which was subsequently oxidized to $H_2VO_4^-$ [16,29]. So the oxidized species $[VO_2(ma)_2]^-$ or $H_2VO_4^-$ was most likely transported via the anion channel, which was inhibited by DIDS (Fig. 1).

Since the changes in oxidation state may play a pivotal role in the biological effects of vanadium compounds, their relative stability, especially in biological media, toward oxidation is important. As indicated by EPR studies, the decay rate of $VO(ma)_2$ is significantly more rapid than $VO(acac)_2$ in buffer solutions, but in the membrane suspensions, the difference between them more or less decreased. The results suggest that under physiological medium, the oxidation of $VO(ma)_2$ and $VO(acac)_2$ are retarded. Sakurai's group also reported that vanadyl species were relatively stable in the blood [9]. So it is reasonable to assume that the +4 oxidation state is stabilized by binding to erythrocyte membrane constituents, leading to the prolonged lifetime of $VO(ma)_2$ and $VO(acac)_2$. This is in accordance with other group's research. Willsky's group indicated that formation of V–protein complexes with abundant serum proteins might affect the available pool of V for biological effects [30]. Recently, Makinen and Brady [8] suggested that the enhanced insulin-mimetic action of organic chelates of $VO^{2+}$ may be dependent on adduct formation with BSA and possibly other serum transport proteins. So we may propose that in the biological media $VO(ma)_2$ and $VO(acac)_2$ can be stabilized in the +4 oxidation state by binding with the biological molecules thus the biopotency increased.

In summary, the uptake kinetics of $VO(ma)_2$ is slower and the accumulation in the cell is somewhat less than $VO(acac)_2$. In addition, $VO(ma)_2$ is more prone to oxidation than $VO(acac)_2$, i.e. $VO(acac)_2$ shows more stable redox property. The higher potency of $VO(acac)_2$ than $VO(ma)_2$ can be thus partly explained.

The difference among these vanadium compounds in their toxicity is also an important factor determining their therapeutic success. As the vanadium complexes contact the cell, the membrane molecules are the frontier targets. The three vanadium compounds more or less increased the fluorescence polarization, indicating the increased order of structure and the decreased fluidity. Comparing with the vanadate ion, the hydrophobic species, $VO(acac)_2$ and $VO(ma)_2$, exerted a stronger effect on the microviscosity of the erythrocyte membranes. This might be related to the hydrophobic interaction with the membrane molecules in the course of cellular uptake. The diffusion of a hydrophobic molecule is a process comprising continuous hydrophobic interaction with the membrane and continuous perturbation on membrane structure. However, the stronger effect on the microviscosity does not imply greater damage to the membrane, because it would be a transient hydrophobic interaction. Once entered into the cell, the vanadium compounds can affect the membrane proteins from the cytoplasmic side. The alterations in the secondary structure of proteins were revealed in FT-IR structure. As shown in Table 2, in the presence of vanadium complexes, the FT-IR spectra are characterized by increased β-sheet content. The increased β-sheet content has been related to the formation of a higher order secondary structure of the membrane proteins [31,32]. It is tempting to suggest that the structural change of the membrane proteins induced by vanadium complexes increased the stability of the membrane and decreased the osmotic fragility of the erythrocytes. In addition, it was reported that vanadate species could bind to the anion channel, the band-3 protein, oxidize the residue sulfhydryl groups of membrane proteins and decrease the fluidity of erythrocyte membrane [33]. The previous studies also showed that glyceraldehyde-3-phosphate dehydrogenase and several other glycolytic enzymes bind to the acidic N-terminus of the cytoplasmic domain of the erythrocyte membrane protein band-3 and inhibit the glycolytic enzymes [34]. So band-3 protein probably participates in control of red cell glycolysis. The alterations in the secondary structure of the membrane proteins should deal with the band-3 protein and this may be another factor to affect the biopotency of the vanadium compounds. Of course, the involvement of the membrane phospholipids cannot be ruled out. Vanadium–membrane interaction is also of concern to the possible toxicity of vanadium compounds. The results showed that $NaVO_3$ might have greater effects on the membrane stability and membrane structure than the vanadium complexes. This may explain the lower toxicity of the complexes than simple salts.

In conclusion: (1) $VO(acac)_2$ and $VO(ma)_2$ were shown to enter the cells mainly by passive diffusion and possess more rapid uptake kinetics and higher intracellular accumulation than $NaVO_3$, which is transported via the anion channel. In view of bioavailability, this behavior might be the determinant for the insulin-mimic potency of vanadium compounds. (2) In physiological fluids, the binding of proteins may play an important part in stabilizing the vanadyl compounds against oxidation. In membrane suspensions, the oxidation of $VO(ma)_2$ and $VO(acac)_2$ can be retarded, i.e. the lifetime and the biological effects of $VO(ma)_2$ and $VO(acac)_2$ may be prolonged. (3) The binding of vanadium to the membrane was shown to lead to a conformation change of the membrane proteins and also to changes in membrane structure. These changes would contribute to their insulin-mimic mechanisms and toxicity.

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