239. Intramolecular Transfer of Excitation Energy, Complexation with Metal Ions, and Interactions with Liposomes of Cyclo[tryptophyl-sarcosyl-sarcosyl-N^{ε}-dansyllysyl-prolyl]

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Summary

A cyclic pentapeptide containing an energy donor group and an energy acceptor group within the molecule, cyclo[Trp-Sar-Sar-Lys(DNS)-Pro], was synthesized. Its conformation, complexation with metal ions, intramolecular energy transfer, and interaction with liposomes were investigated. The precursor, cyclo[Trp-Sar-Sar-Lys(Z)-Pro], contained one *cis*-sarcosyl peptide bond and a β-turn in which the Pro-residue occupied the 3rd position. This cyclic pentapeptide formed a complex selectively with Ba^{2+} and its binding constant in 95% aqueous methanol solution was 2.0×10^3 l · mol^{-1}. The intramolecular energy-transfer efficiency of cyclo-[Trp-Sar-Sar-Lys(DNS)-Pro] was almost constant between $-60°$ and $40°$ in ethanol, while it increased at temperatures lower than 34° in liposomes as a result of the pre-transition of the lipid assembly. On the other hand, the intramolecular energy-transfer efficiency of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] decreased upon complexation with Ba^{2+}.

Introduction. – To clarify the relationship between structure, conformation, and various functions of peptides, synthetic cyclic peptides have been used as models and their conformations and functions have been studied by spectroscopic methods [1-4]. For example, information about the mechanism of ionophoric action, was gained by X-ray diffraction, circular dichroism (CD.), and nuclear magnetic resonance (NMR.) spectroscopy [5-13]. From our investigations with a series of cyclic octapeptides [14], we have found that the conformation is strongly solvent-dependent. Therefore, to elucidate the mechanism of the action of peptides on membranes, it is necessary to carry out the conformational analyses in lipid membranes. Some studies of peptides in liposomes and micelles have already been carried out by CD. and NMR. spectroscopy [15] [16]. However, in these investigations the probe concentrations employed were very high, possibly producing artefacts due to peptide aggregation and membrane perturbation. On the other hand, fluorescence methods are effective at low probe concentrations, thus avoiding such difficulties. We therefore attempted to synthesize the cyclic pentapeptide cyclo[Trp-Sar-Sar-Lys(Z)-Pro] containing a Trp-residue as a fluorescent probe,

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and to analyze its conformation and various interactions by fluorescence in addition to CD. and NMR. This cyclic pentapeptide contains three *N*-substituted peptide bonds, which make the conformation versatile due to the *cis/trans*-isomerization. It was expected to form stable complexes with metal ions. Its interaction with artificial membranes was investigated by measuring the fluorescence of the Trp-residue.

The dynamic properties of peptide chains have been investigated by measuring the energy-transfer efficiency between an energy donor and an energy acceptor which were introduced to both ends of a linear molecule [17]. In this study cyclo-[Trp-Sar-Sar-Lys(DNS)-Pro] was synthesized, in which a Trp-residue, the energy donor, and a 5-dimethylaminoaphthalenesulfonyl (DNS) group, the energy acceptor, are present in the same molecule. The intramolecular transfer of excitation energy occurring in the cyclic peptide may be affected by structural changes of cyclic skeleton and the mobility of the side chains, thus affording information about the conformation.

In multifunctional molecules the elementary functions tend to cooperate with each other; for example, a cyclic hexapeptide, cyclo(D-Leu-Glu-His)$_2$, is an asymmetric catalyst of the hydrolysis of α-amino-acid *p*-nitrophenyl ester only in the form of its Cu^{2+}-complex [18]. Bis-crown ethers, in which the two crown ether moieties are covalently bound by an azobenzene group, are ligands for complex formation with metal ions, and the stability of such complexes can be regulated by *cis/trans*-photoisomerization of the azobenzene group [19]. The cyclic pentapeptide, cyclo-[Trp-Sar-Sar-Lys(DNS)-Pro], is provided with two functions: the complex formation with metal ions and the intramolecular energy transfer. It should be interesting to know how the intramolecular energy transfer is affected by the complexation with metal ions.

Experimental. – 1. *Synthesis.* The synthetic route to cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] is shown in *Figure 1.* The Trp-residue was protected by the formyl group to avoid oxidation during the acid treatment [20].

Cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro]. Boc-Trp(CHO)-Sar-Sar-Lys(Z)-Pro-OH was synthesized by stepwise elongation from the C-terminus in liquid phase. To the solution of Boc-Trp(CHO)-Sar-Sar-Lys(Z)-Pro-OH (2.1 g) and *N*-hydroxysuccinimide (HOSu, 0.44 g) in cold dimethylformamide (DMF), dicyclohexylcarbodiimide (DCCI, 0.63 g) was added at 0°. After stirring the solution overnight, the solvent was removed under reduced pressure. To the residue, ethyl acetate containing a few drops of acetic acid was added and the precipitate was filtered off. The filtrate was washed with water and dried (Na_2SO_4). The solvent was evaporated to dryness, anisole (1 ml) and trifluoroacetic acid (TFA, 15 ml) were added at 0°. After 30 min the solution was concentrated to a small volume and treated with dry ether. The white precipitate was collected and dried over NaOH under reduced pressure. This product, the TFA-salt of Trp(CHO)-Sar-Sar-Lys(Z)-Pro-OSu, was dissolved in DMF containing a few drops of acetic acid; the solution was then added dropwise to pyridine at 30°. After the reaction, the solvent was evaporated to dryness, the residue dissolved in $CHCl_3$, and the solution purified by gel-permeation chromatography using a *JASCO MEGAPAK GEL 201* column. Further purification was carried out by a silica gel column using ethyl acetate/CH_3OH 3:1 (*v/v*) as eluent. The product was dissolved in a small amount of ethyl acetate, and precipitated with hexane to obtain cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro]. Yield from Boc-Try(CHO)-Sar-Sar-Lys(Z)-Pro-OH: 35%, TLC. in $CHCl_3$/CH_3OH/acetic acid 95:5:3, Rf(I) 0.10; butanol/acetic acid/H_2O 65:10:25, Rf(II) 0.25; ethyl acetate/CH_3OH 4:1, Rf(III) 0.22.

$C_{37}H_{45}O_8N_7 \cdot \frac{1}{2} H_2O$	Calc.	C 61.31	H 6.40	N 13.53%
(724.82)	Found	„ 61.44	„ 6.58	„ 13.28%

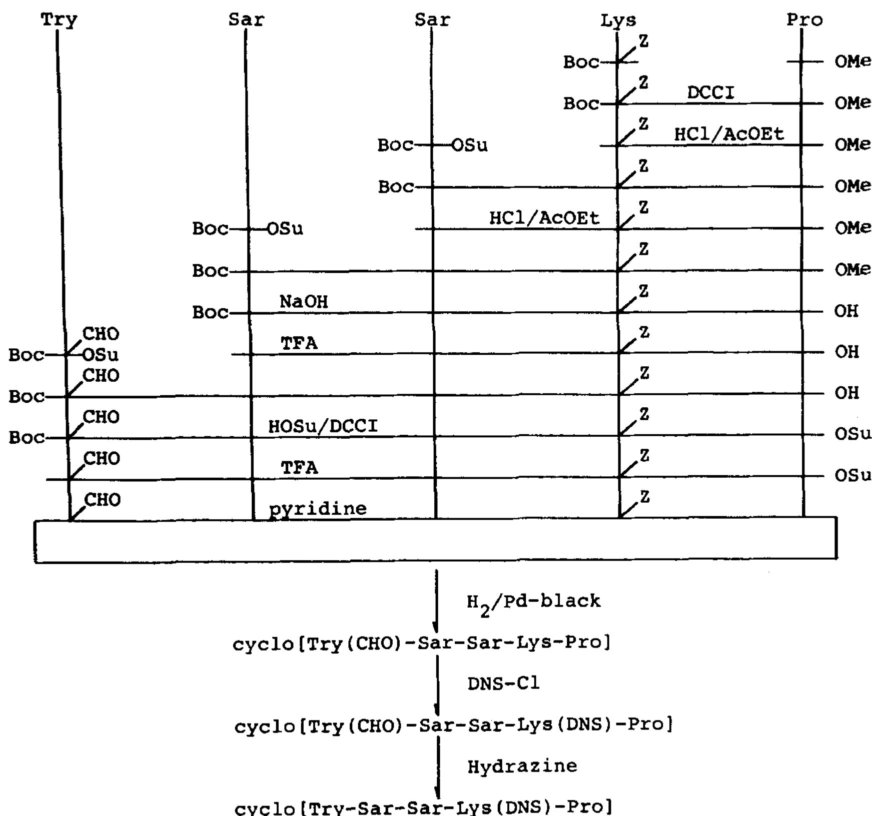

Fig. 1. *Synthetic route to the cyclic pentapeptide cyclo[Trp-Sar-Sar-Lys(DNS)-Pro]*

Cyclo[Trp-Sar-Sar-Lys(DNS)-Pro]. Cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro] (0.173 g) was dissolved in CH_3OH containing acetic acid (17 µl), and hydrogenated using Pd as a catalyst. The catalyst was filtered off and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in $CHCl_3$, and treated sequentially with triethylamine (81 µl) and DNS chloride (78 mg) in $CHCl_3$. The solution was then concentrated and eluted through a gel-permeation chromatography column to obtain cyclo[Trp(CHO)-Sar-Sar-Lys(DNS)-Pro]. This compound (88 mg) was dissolved in a mixture of DMF (1.4 ml) and hydrazine monohydrate (0.135 ml), and the solution was left standing for 48 h [21]. Sufficient water was then added to cause precipitation. The precipitate was gathered and washed with water. It was purified by gel-permeation chromatography. The pure cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] thus obtained showed one spot on TLC: Rf(II) 0.28.

$C_{40}H_{50}O_7N_8S$	Calc.	C 61.05	H 6.40	N 14.24	S 4.07%
(786.95)	Found	„ 60.76	„ 6.61	„ 14.02	S 3.88%

Cyclo[Trp-Sar-Sar-Lys(Z)-Pro] was obtained from cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro] by analogous hydrazine treatment.

2. *Preparation of liposomes.* Dipalmytoyl phosphatidylcholine (DPPC) was purchased from *Fluka AG.* The lipid was dispersed in 10 mM phosphate buffer (pH 7.1) containing 0.1 M NaCl, and was sonicated and ultracentrifuged at 100,000 g to obtain liposomes. The fluorescent probe was added to the liposome preparation as an ethanol solution. 2-(9-Anthroyloxy)stearic acid (2-AS) was purchased from *Molecular Probes Inc.*

3. *Measurements.* Absorption, fluorescence, CD., IR., and NMR. measurements were carried out on a *Shimadzu UV 210* spectrophotometer, a *Hitachi MPF-4* spectrofluorometer, a *JASCO J-20* spectropolarimeter, a *JASCO A202* diffraction-grating IR. spectrophotometer, and a *JEOL FX900 Fourier* transform NMR. spectrometer, respectively. The chemical shifts of ^{13}C-NMR. spectra were obtained using solvent signals as standard (CD_3OD: 48.0 ppm, CD_3CN: 1.3 ppm, $CDCl_3$: 76.9 ppm).

Fluorescence spectra were obtained by excitation at 281 nm. Fluorescence excitation spectra were obtained by monitoring the fluorescence from the DNS-group at 520 nm. 100%-energy transfer was determined from the absorption spectrum of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro]. 0%-transfer was

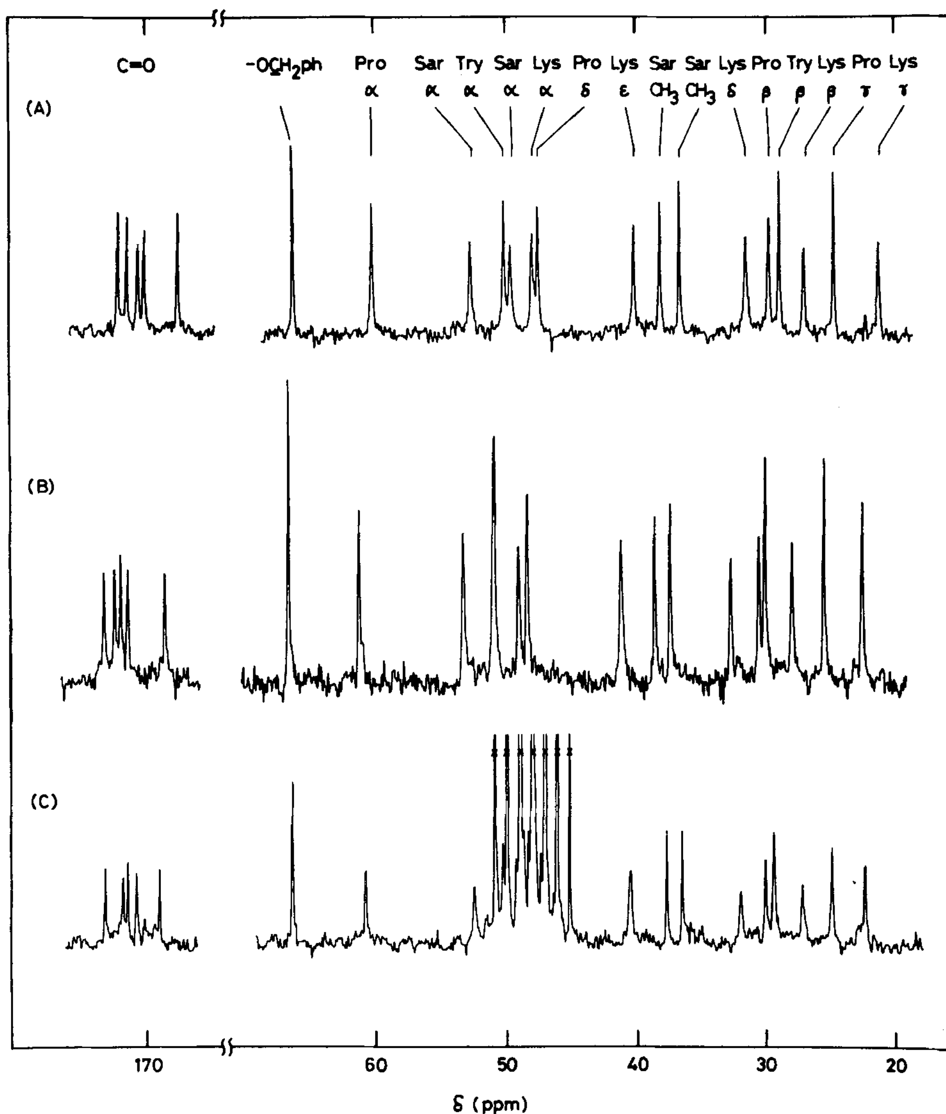

Fig. 2. *^{13}C-NMR. spectra of cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro]*
(A: in $CDCl_3$, B: in CD_3CN, and C: in CD_3OD, x means the signal of solvent)

estimated from the excitation spectrum of cyclo[Trp(CHO)-Sar-Sar-Lys(DNS)-Pro], because the N^i-formyltryptophan is practically non-fluorescent. Both absorption and excitation spectra were normalized with the absorption intensity at the wavelength of the maximum absorption of DNS-group. The energy-transfer efficiencies were calculated from the intensity at 290 nm of Trp in the excitation spectra of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro].

Results and discussion. – 1. *Conformation of cyclo [Trp (CHO)-Sar-Sar-Lys (Z)-Pro].* ^{13}C-NMR. spectra of cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro] in $CDCl_3$, CD_3CN, and CD_3OH are shown in *Figure 2*. The assignment of each signal in $CDCl_3$, as indicated in *Figure 2A*, is based on chemical shift and the off-resonance ^{1}H-^{13}C-double resonance experiments. Only one signal appeared for each C-atom, indicating that this cyclic pentapeptide populated only one conformation in these solvents on the NMR. time scale. Comparison of these spectra suggested that the conformation in these solvents was the same. On the other hand, in D_6-dimethylsulfoxide (D_6-DMSO) several signals appeared for each C-atom, indicating that several conformations coexist (data are not shown). These conformers were considered to be *cis/trans*-isomers with reference to the *N*-substituted peptide bonds.

In ^{1}H-NMR., the addition of D_6-benzene to the $CHCl_3$-solution of cyclo-[Trp(CHO)-Sar-Sar-Lys(Z)-Pro] induced the shifts of sarcosyl $N-CH_3$ signals as shown in *Figure 3*. The signal shifted strongly to higher magnetic field should be ascribed to the $N-CH_3$ protons adjacent to a *trans*-peptide bond and the signal shifted only slightly to higher magnetic field to those adjacent to a *cis*-peptide bond [22]. This consideration led to the assignment of the signals appearing at 36.6 and

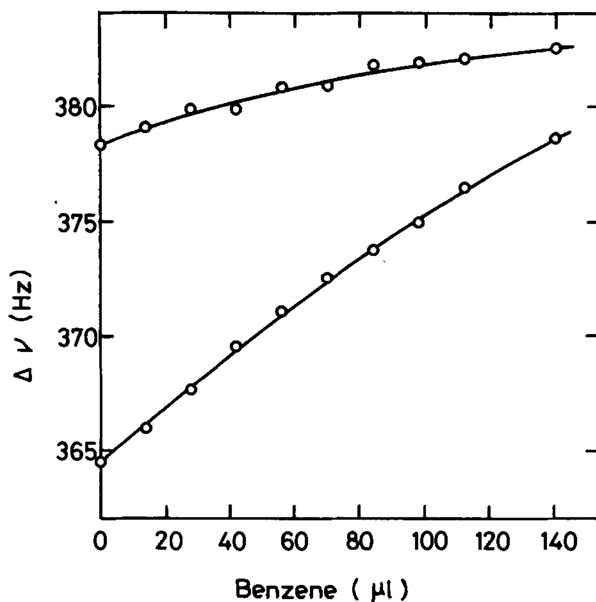

Fig. 3. *Change of the chemical shifts of $N-CH_3$ signals of cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro] with the addition of D_6-benzene in $CDCl_3$* ($\Delta\nu$ represents the difference of the frequencies of $N-CH_3$ signal from phenyl signal as standard)

38.1 ppm in the ^{13}C-NMR. spectrum *(Fig. 2A)* to $N-CH_3$ C-atoms adjacent to a *trans*- and a *cis*-peptide bond, respectively, by the selective irradiation of each class of $N-CH_3$ protons in the ^{13}C-NMR. spectrum.

Signals of Pro-C^{β}- and Pro-C^{γ}-atoms were located in the usual region for *trans*-peptide bonds [2]. Thus, all the peptide bonds in this cyclic peptide were *trans* with the exception of one *cis*-Sar-peptide bond.

The temperature dependencies of the chemical shifts of NH-signals in CD_3CN were found to be 7.3×10^{-3} ppm · deg^{-1} for the urethane amide proton, and 3.0×10^{-3} and 3.1×10^{-3} ppm · deg^{-1} for the other amide protons. The IR. spectrum of cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro] in $CHCl_3$ showed a strong absorption at 3294 cm^{-1}, the extinction coefficient being independent of the concentration. Therefore, the amide protons of the cyclic skeleton appeared to be involved in intramolecular H-bonds [23] [24]. As proton acceptors for the H-bonded amide protons, five carbonyl groups of the cyclic skeleton and of the urethane group of the side chain were considered. Inspection of the *Corey-Pauling-Koltun* (CPK.) molecular model revealed that the latter carbonyl group can form an intramolecular H-bond with Lys-NH, but not with Trp-NH. β- or γ-Turns are often found in cyclic peptides with intramolecular H-bonds. If a γ-turn ($1 \leftarrow 3$ intramolecular H-bonding) was assumed, the structure could be represented schematically as (A) or (B) in *Figure 4.* In either case a Pro-residue would be involved in the γ-turn. For such structures, the signal of Pro-C^{β}-atom in ^{13}C-NMR. spectra has been reported

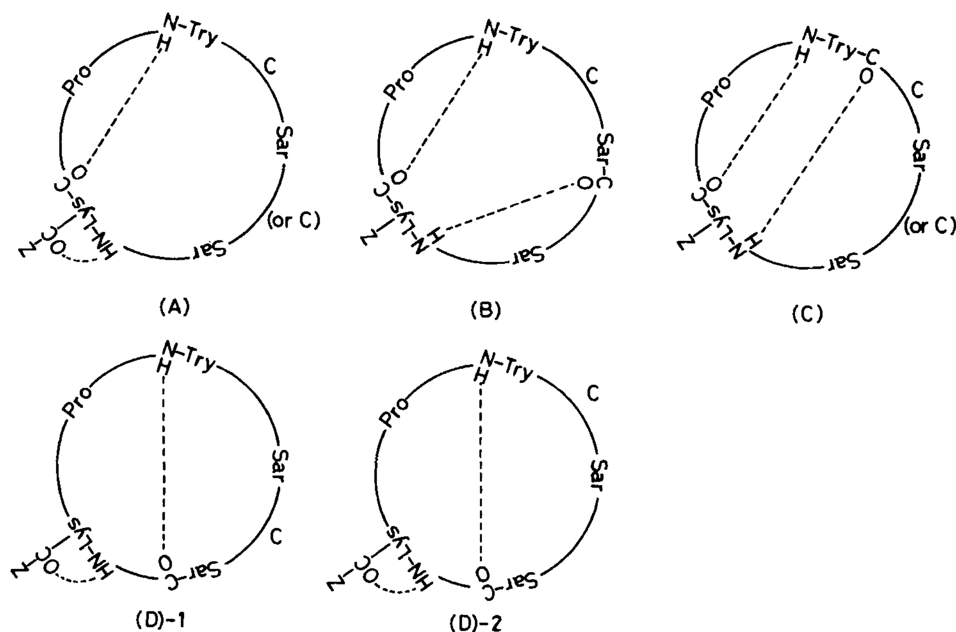

Fig. 4. *Possible conformations of cyclo[Trp(CHO)-Sar-Sar-Lys(Z)-Pro] with two amide protons forming intramolecular H-bonds* (dotted line) *of β-turn-type, γ-turn-type, or with the urethane group* (C represents a *cis*-peptide bond)

to be shifted to higher magnetic fields [2]. This was not observed in the present case, so that the structures (A) and (B) in *Figure 4* are not likely. However, if a β-turn ($1 \leftarrow 4$ intramolecular H-bonding) occurs in the present case, the structures (C) or (D) might be considered. The conformation of (C) contains a *cis*-peptide bond in the β-turn. The simultaneous formation of an intramolecular H-bond with Trp-NH would lead to the formation of the same γ-turn as shown in (B). So the structure (C) is also unlikely. In the structure (D) the Pro-residue takes the 3rd position in the β-turn, which has been rarely found in nature. Since the structure (D)-2 is far less sterically hindered than that of (D)-1, the former is the most plausible structure of all. This conformation is characterized as follows: *i)* it contains a β-turn in which the Pro-residue is in the 3rd position, *ii)* intramolecular H-bonding is formed between the carbonyl group of the side chain and the NH of the cyclic skeleton (the same is found in ferrichrome [25]), and *iii)* one *cis*-peptide bond is contained in the ring structure (this is observed in cyclo(Ala-Pro-Gly-D-Phe-Pro) [16]). The above discussion is based on the assumption of the formation of $1 \leftarrow 3$ or $1 \leftarrow 4$ intramolecular H-bonds. The occurrence of other structures than those considered here cannot be excluded.

2. *Complexation of cyclo[Trp-Sar-Sar-Lys(Z)-Pro] with metal cations.* Complexation of cyclic peptides with metal ions has been correlated with the flexibility of ring structure [1]. Cyclo[Trp-Sar-Sar-Lys(Z)-Pro] contains three *N*-substituted peptide bonds, which make *cis*- as well as *trans*-configurations possible. Therefore, this cyclic pentapeptide is expected to bind metal ions efficiently due to the number of available conformations. On the other hand, it could be argued that the ring size is too small to provide the cyclic pentapeptide with a sufficient flexibility for complexation.

With the addition of a 10- to 20-molar excess of KCl or $NaClO_4$ to cyclo-[Trp-Sar-Sar-Lys(Z)-Pro] in 95% aqueous methanol solution, no change of the CD. spectra was observed, giving no proof of complexation. On the other hand, with the addition of $Ba(ClO_4)_2$, the CD. spectra changed as shown in *Figure 5*, indicating complex formation.

From the variation of CD. spectra, the stoichiometric composition of the complex was calculated to be 1:1 and the equilibrium constant of the complex formation 2.0×10^3 l · mol^{-1} [26].

Addition of $Ca(ClO_4)_2$ also caused a similar change in CD. spectra, but the equilibrium constant was as small as 10 l · mol^{-1}. Therefore, cyclo[Trp-Sar-Sar-Lys-(Z)-Pro] selectively formed a complex with Ba^{2+}.

In acetonitrile, precipitation occurred upon addition of $Ba(ClO_4)_2$. ^{13}C-NMR. spectra of the white precipitate dissolved in CD_3OD showed evidence for the co-existence of different conformations. However, it was not possible to identify the conformers.

3. *Intramolecular excitation-energy transfer of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro].* The temperature dependence of the intramolecular energy-transfer efficiency of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] in ethanol is shown in *Figure 6.* The efficiency of the energy transfer is about 82% between 0 and 40°. A slight increase of the transfer efficiency was observed at lower temperatures. Since the absorption spectra of this cyclic pentapeptide hardly changed with temperature, the increase of the

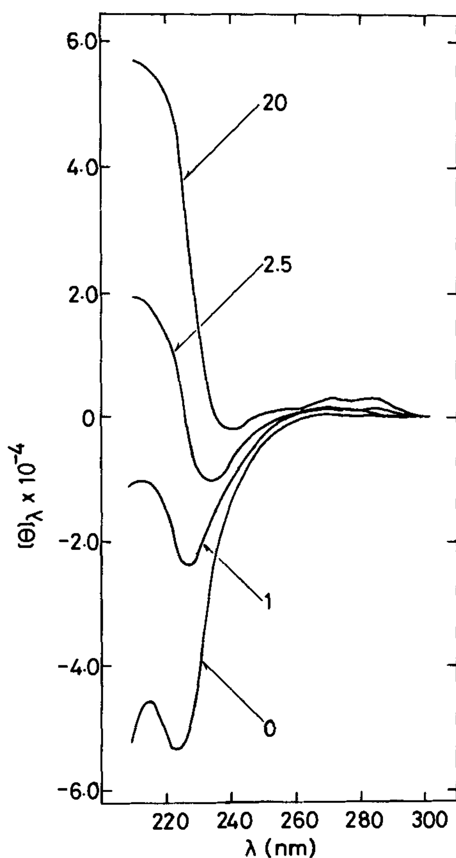

Fig. 5. *Change of CD. spectra of cyclo[Trp-Sar-Sar-Lys(Z)-Pro] in MeOH/H_2O 95:5* (v/v) *with the addition of Ba(ClO_4)$_2$* (The numbers of the curves represent the ratio of [Ba^{2+}]/[cyclic peptide])

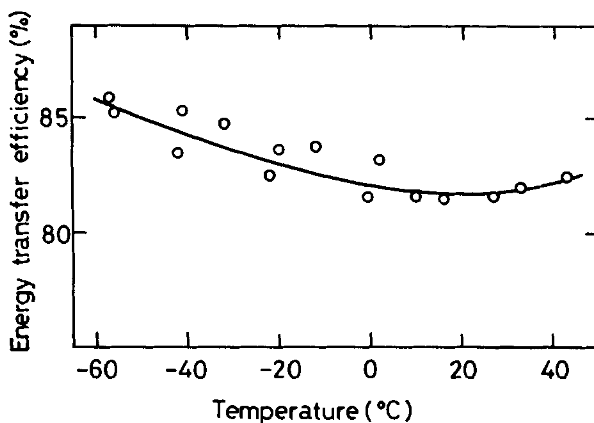

Fig. 6. *Temperature dependence of intramolecular energy transfer of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] in ethanol* ([cyclic peptide] = 1.0×10^{-5}M)

transfer efficiency at lower temperatures may be due to the increase of the quantum yield of the Trp-residue.

The critical distance for the energy transfer of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] in ethanol was estimated to be 2.1 nm from the fluorescence spectrum of cyclo-[Trp-Sar-Sar-Lys(Z)-Pro] and the excitation spectrum of cyclo[Trp(CHO)-Sar-Sar-Lys(DNS)-Pro] [27]. Assuming that the conformation of the ring structure of cyclo-[Trp-Sar-Sar-Lys(DNS)-Pro] is the same as that of cyclo[Trp-Sar-Sar-Lys(Z)-Pro] ((D)-2 in *Fig. 4*), the distance between the indolyl and the DNS-groups ranges from 0 to 2.0 nm. This large variation can be explained by the flexibility of the Lys side chain. Although both chromophores are situated within the critical distance for energy transfer, the efficiency of energy transfer was always lower than 100%. The possible reason for this phenomenon could be that the two chromophores do not always assume a mutual orientation suitable for energy transfer during the life-time of the excited indolyl group.

4. *Interaction of cyclo [Trp-Sar-Sar-Lys(DNS)-Pro] with DPPC-liposomes.* The maximum fluorescence of the Trp-indolyl group of cyclo[Trp-Sar-Sar-Lys(Z)-Pro] was observed at 340 nm in the presence of DPPC-liposomes. The maximum fluorescence of the indolyl group in aqueous solution was at 350 nm. Therefore, the blue shift of the fluorescence was taken to indicate that the cyclic peptide was buried into the hydrophobic region of the DPPC-liposomes [28]. The excitation of cyclo[Trp-Sar-Sar-Lys(Z)-Pro] added to DPPC-liposomes containing 2-AS was followed by energy transfer from the cyclic peptide to 2-AS. This fact also verified the interaction of the cyclic peptide with the membrane. The efficiency of the energy transfer (under the condition that [cyclo[Trp-Sar-Sar-Lys(Z)-Pro]] = 4.7×10^{-6} M; [2-AS] = 3.6×10^{-6} M, [DPPC] = 1 mM) was about 4% in the temperature range for the liquid crystalline state of the membrane, while it increased to 11% in the phase-transition region where the membrane enters the crystalline state. This phenomenon was attributed to local increases of the surface density of the acceptor, because the phase separation at the phase-transition temperature produces crystalline regions excluding the probes and liquid domains containing higher concentrations of probes [29].

The temperature dependence of the intramolecular energy-transfer efficiency of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] in DPPC-liposomes is shown in *Figure 7:* a rather sharp transition was found at about 34°, with a definitely larger increment of energy-transfer efficiency at lower temperatures. Since the intramolecular energy transfer in ethanol was almost constant above 0°, the increase below 34° must have been caused by the interaction of the cyclic peptide with DPPC-liposomes, in which phase transitions occur at 42° and 34°. The former is related with the packing of alkyl groups of the lipid molecules and the latter with the mobility of methyl groups of the polar region of the lipid molecules [30]. Therefore it is likely that cyclo-[Trp-Sar-Sar-Lys(DNS)-Pro] is located near the polar head groups of lipid molecules and the pre-transition of the membrane makes the orientation of indolyl and DNS-groups favorable for energy transfer. The present result is an example showing that the structural change of membranes during phase transition can affect the conformation of membrane peptides.

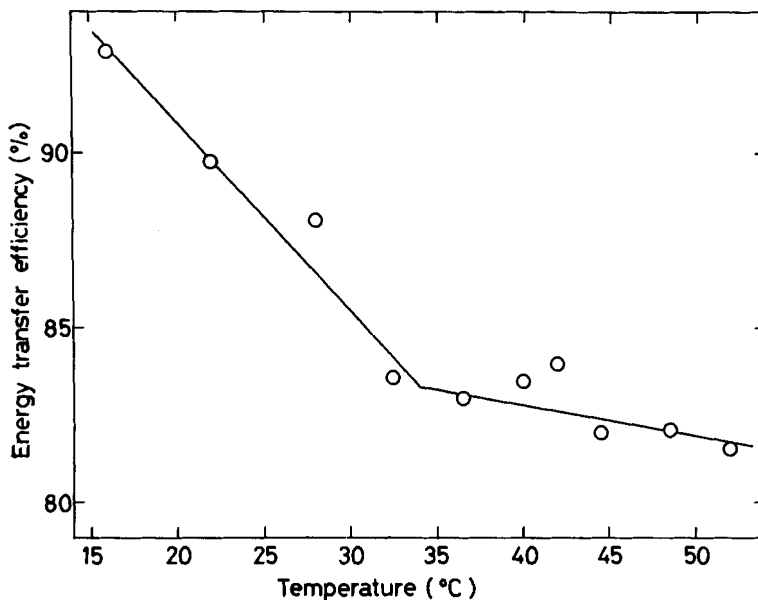

Fig. 7. *Temperature dependence of intramolecular energy transfer of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] in DPPC-liposome solution* ([cyclic peptide] = 5.25×10^{-6}M)

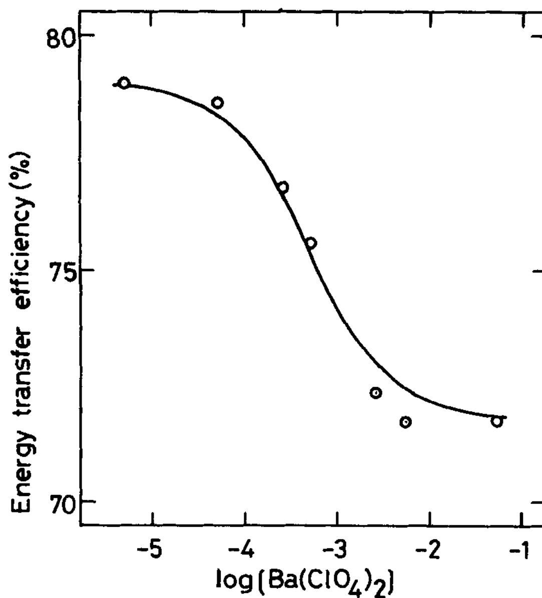

Fig. 8. *Dependence of intramolecular energy transfer of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] on the concentration of Ba^{2+} in $MeOH/H_2O$ 95:5* (v/v) (For the calculation of the theoretical value, see text; [cyclic peptide] = 5.25×10^{-6}M)

5. *Control of intramolecular energy transfer by complexation with Ba^{2+}-ion.* The function of cyclo[Trp-Sar-Sar-Lys(DNS)-Pro] is two-fold: it forms a complex with Ba^{2+}-ions and it is capable of intramolecular energy transfer. *Figure 8* shows the change of the intramolecular energy-transfer efficiency caused by addition of Ba^{2+}. The energy-transfer efficiency began to decrease at about 10^{-4}M Ba^{+} and ultimately became stable at about 72% at 10^{-2}M Ba^{2+}. The theoretical efficiency of energy transfer was calculated as follows. The efficiencies of energy transfer of the cyclic pentapeptide were taken as 79% for the metal-ion-free state and 71.8% for the complexed state. The fractions of free and complexed cyclic peptides at various Ba^{2+}-concentrations were calculated using the equilibrium constant obtained from CD.-spectra. The energy-transfer efficiencies at various Ba^{2+}-concentrations are represented by the curve in *Figure 8.* The observed values are in good agreement with the calculated curve. Therefore, the decrease of the intramolecular energy-transfer efficiency by Ba^{2+} can be ascribed to the complex formation of the cyclic peptide with Ba^{2+}. This assignment is supported by the observation that the addition of Ba^{2+} to the 95% aqueous methanol solution of cyclo[Trp-Sar-Sar-Lys-(Z)-Pro] caused a blue shift of the maximum fluorescence and a slight increase of the quantum yield. The critical distance of the energy transfer calculated on the basis of the fluorescence spectrum was almost the same as that in the absence of Ba^{2+}. Therefore, the decrease of the energy-transfer efficiency due to the complex formation with Ba^{2+} should be ascribed to a different orientation of the chromophores. This could be caused by the effect of complexation on ring conformation.

The effect of this conformational change upon the intramolecular energy-transfer efficiency was not very large *(Fig. 8).* This is plausible, because a conformational change of the cyclic skeleton should not seriously affect the orientation of chromophores, as the DNS-group is bound to the end of a very flexible side chain.

A cyclic peptide, in which an energy donor and an energy acceptor are located close to the cyclic skeleton and the distance between them is close to the critical distance of energy transfer should be very interesting, because with this type of cyclic peptide a remarkable change of the intramolecular energy-transfer efficiency with complexation is expected. In this case, the energy-transfer efficiency might also be controlled by the change of membrane structure.

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