*Immunology* 1990 **69** 323–328

# Location of membrane-bound hapten with different length spacers

K. KIMURA,* Y. ARATA,* T. YASUDA,† K. KINOSITA‡ & M. NAKANISHI§ *Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, †Institute for Environmental Medicine, Okayama University Medical School, Misasa, Tottori, ‡Department of Physics, Keio University, Hiyoshi, Minato-ku, Yokohama and §Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya, Japan

*Accepted for publication 20 September 1989*

## SUMMARY

Immunogenic activity of a lipid hapten is strongly dependent on the length and nature of the linker chain (spacer) connecting the hapten to the head group of the lipid. A derivative containing a very short or a long spacer is known to be less effective for antibody binding than that of an intermediate length. In the present experiment, this was confirmed first by experiments of antibody binding to TNP lipid haptens with different length of spacers and of antibody-dependent macrophage binding to them. Second, we determined the location of the TNP haptens in lipid bilayer membranes by fluorescence energy transfer. It was found that vertical distances between TNP groups (acceptors), which were assumed to be randomly distributed in a plan parallel to the membrane surface, and a pyrene fluorophore (donor), which was embedded in the middle of lipid membranes, were 10·2–10·5 Å in the DMPC membranes and 13·2–13·9 Å in the DPPC membranes. The vertical distances were about 3 Å longer in the DPPC membranes than in the DMPC membranes. However, they were almost independent of the length of spacers. This indicates that TNP residues of the lipid haptens locate at the similar vertical position on the membrane surfaces even if they have different length spacers. From these results we suggested that the affinity of the spacer groups to the bilayer surfaces can modulate the binding affinity of antibody to lipid hapten on the membrane surfaces. This was partly supported by the binding experiments of TNP spacers to the bilayer membranes.

## INTRODUCTION

During the past 20 years numerous studies have been performed on the immunology of liposomes. The issue of lipid-like epitopes and immune response is practical in considering tumour-specific antigens, etc. Liposomes have often served as model systems in these studies (Six, Uemura & Kinsky, 1973; Brûlet & McConnell, 1977; Dancey, Isakson & Kinsky, 1979; Balakrishnan, Mehdi & McConnell, 1982; Alving & Richards, 1983; Ho & Huang, 1985). These studies have revealed that immunogenic activities of the hapten are dependent on many variables. They are the chemical composition of liposomes, the density of hapten groups, the chemical nature of the hapten, and the length of the linker chain (spacer). In the previous studies, however, no one has determined yet how the antibody bindings are affected by the transmembrane location of lipid hapten. Thus, in this paper we have tried to determine the vertical location of the membrane bound hapten.

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidyl-choline; FCS, fetal calf serum; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; TNP, 1,3,5-trinitrophenyl.

Correspondence: Dr M. Nakanishi, Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan.

It has been shown that derivatives containing very short or long spacers are less effective for antibody binding and triggering than those of the intermediate chain length (Brûlet & McConnell, 1977; Dancey, Isakson & Kinsky, 1979; Balakrishnan, Mehdi & McConnell, 1982). Several explanations have been given for this kind of phenomenon. One of these explanations is that the major source of the difference arises from differences in accessibility. That is, by lengthening the spacer residue, the hapten moiety is extended further away from the liposomal bilayer. This interpretation, however, neither explains the decrease in immunogenicity nor in antibody binding for the case of longer spacers. Another interpretation for the decrease in immunogenicity and in antibody binding for the case of longer spacers is that the haptenic group has become buried within the bilayer when longer-spacer phospholipids are used.This can inhibit the binding of specific antibody. Until now, however, none of the physical evidence has been given to answer the question.

Answering the above question is important to understand the general problem of cell surface recognition in immunology and in membrane biochemistry. so, we have studied here precise locations of the membrane-bound TNP haptens with different length spacers by fluorescence energy transfer method (Forster, 1965; Stryer, 1978; Thomas, Carlsen & Stryer, 1978; Kleinfeld & Lukacovic, 1985) By applying this method to the TNP lipid

hapten with different length spacers, the location of the TNP hapten in the membrane have been determined. The results made it possible to understand how the different length of spacers can modulate the efficiency of antibody binding and triggering.

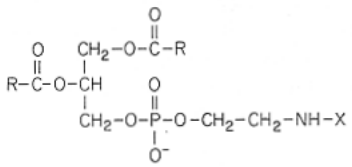
## MATERIALS AND METHODS

*Materials*

L-α-Dimyristoylphosphatidylcholine (DMPC) and L-α-dipalmitoyl-phosphatidylcholine (DPPC) were purchased from Avanti (Birmingham, AL). 1, 3, 5-trinitrophenyl (TNP) lipid haptens with different length spacers were prepared by the previous method (Okada *et al.*, 1982). The structures of TNP-lipid haptens with different length spacers are shown in Fig. 1. 1-Pyrenedodecanoic acid was purchased from Molecular Probe (Junction City, OR). Preparation of mouse monoclonal IgG (IgG1, IgG2a and IgG2b) against TNP residues was described in the previous paper (Kimura *et al.*, 1986). Binding constants between anti-TNP–IgGs and TNP–amino-caproylic acid were $1{\cdot}0–5{\cdot}0 \times 10^7$/M.

*Liposomes*

Single unilamellar vesicles for the energy transfer measurements were prepared by the injection of an ethanolic solution of a mixture of phospholipid (DMPC or DPPC), 1-pyrenedodecanoic acid and a TNP–lipid hapten into a phosphate-buffered saline (PBS, pH 7·2) at 70° (Batzri & Korn, 1973; Kremer & Esker, 1977). Single unilamellar vesicle preparation was checked by the trapped umbelliferone phosphate method (Six *et al.*, 1974). Multilamellar liposomes were prepared by co-dissolving phospholipid and the TNP–lipid hapten in chloroform and drying onto the sides of flasks. Then, PBS was added to the flasks which were kept at 70° for 15 min and were shaken to give a liposome suspension. We described supported monolayer preparations in a previous paper (Kimura & Nakanishi, 1985). The density of hapten on the membrane surface was checked by the following method. As for single unilamellar vesicles we collected single unilamellar fractions by gel chromatography. Then, we condensed vesicle fractions and determined the concentrations of TNP–lipid and DMPC (or DPPC) in the vesicles. The density of lipid hapten in vesicles was the same for all spacer chains tested here. As for multilamellar liposomes we spun down the liposomes and determined the concentrations of TNP–lipid and DMPC (or DPPC). The density of lipid hapten in multilamellar liposomes was also the same for all spacers.



| | No. carbon atoms in spacers (TNP–$C_n$–PE) | |
|---|---|---|
| TNP-PE | 0 | X = -TNP |
| TNP-*N*-propyl-PE | 3 | $-CO-(CH_2)_2-NH-TNP$ |
| TNP-*N*-butyryl-PE | 4 | $-CO-(CH_2)_3-NH-TNP$ |
| TNP-*N*-caproyl-PE | 6 | $-CO-(CH_2)_5-NH-TNP$ |
| TNP-*N*-capryl-PE | 8 | $-CO-(CH_2)_7-NH-TNP$ |
| TNP-*N*-lauryl-PE | 12 | $-CO-(CH_2)_{11}-NH-TNP$ |

**Figure 1.** Structure of trinitrophenylated (TNP) phosphatidylethanolamine derivatives containing different length spacers.

*Antibody binding to TNP–lipid haptens*

Antibody binding on the membrane-bound TNP–haptens with different length spacers was measured using multilamellar iposomes. Anti-TNP–IgG was incubated with multilamellar liposomes (DMPC and DPPC) with 1% of TNP–lipid haptens or without them (a control experiment) for 30 min at 4°. After incubation, the liposome–IgG complexes were spun down and tryptophan fluorescence intensities of IgG in the supernatant were measured (excited at 280 nm and observed at 350 nm). They were compared with a value of the control experiment in which we used liposomes without TNP lipid haptens.

*Macrophage-binding experiments*

For macrophage-binding experiments, a 50 μl solution of anti-TNP–IgG (1·5 μM) was first added to supported-DMPC monolayers containing 1% TNP–$C_n$-PE. They were kept at 4° for 30 min. After washing unbound antibody with PBS (+1% FCS), macrophages (J774.1 cell line) in PBS (1% FCS) were introduced between a cover glass and a slide, and allowed to settle on the lipid monolayers. Following incubation at 37° for 15 min, the slide was inverted and examined using a Nikon VFD-R microscope with a camera. The percentages of macrophages bound to the monolayers were calculated by photographing several fields on the slide at random and counting both cells in the focal plane of the monolayers and cells in the focal plane of a bare glass slide (Hafeman, Tscharner & McConnell, 1981; Kimura & Nakanishi, 1985).

*Fluorescence measurements*

Fluorescence spectra were observed with a Hitachi model 650-60 fluorescence spectrophotometer. Decay of fluorescence intensity after pulsed excitation was measured with a single photon counting apparatus as described previously (Kinoshita *et al.*, 1981). Excitation wavelength was 340 nm and the emission above 370 nm was collected at 30°

*Determination of the distance*

The location of the membrane-bound TNP hapten was examined with the pyrene moiety of 1-pyrenedodecanoic acid as the reference point. Here, bound TNP groups were assumed to be randomly distributed in a plane parallel to the membrane surface. The vertical distance (z) between this plane and the pyrene group, presumably situated in the middle of the bilayer membrane, was determined from the analysis of the excitation energy transfer from the pyrene fluorophore (donor) to the TNP groups (acceptors). In this multi-acceptor system, the decay of donor fluorescence after a pulsed excitation is expected to be non-exponential. The initial rate $k_d$ of the decay, however, is related to the distance (z) by a simple equation (Kometani *et al.*, 1987):

$$k_d = \frac{1}{\tau_d} \times \left(1 + \frac{\pi}{3A} \cdot \frac{R_0^6}{z^4}\right) \quad (1)$$

where $\tau_d$ is the fluorescence lifetime of the donor in the absence of acceptors, $R_0$ is the critical distance for energy transfer, and A

is the average membrane area occupied by an acceptor. We assumed that the average membrane area (A) is 6200 Å$^2$ for DMPC and 4450 Å$^2$ for DPPC membranes which contain 1% TNP–lipid haptens (Philip, 1987). The orientations of the donor and acceptors were assumed to be random. This assumption was substantiated at least for the donor, since the anisotropy of pyrene fluorescence dropped to zero within a few nanoseconds after pulsed excitation (indicating rapid rotation). We described the formula more precisely in the previous paper (Kometani *et al.*, 1987).

## RESULTS

### Antibody binding to TNP–lipid haptens with different length spacers

We first checked the binding affinity of anti-TNP IgG2b to TNP–lipid haptens in multilamellar liposomes with different length spacers (Fig. 2). The results showed that a derivative containing a very short (TNP–$C_0$-PE or TNP–$C_3$-PE) or a longer spacer (TNP–$C_{12}$-PE) was less effective for antibody binding than a derivative containing an intermediate length spacer (TNP–$C_6$-PE) as shown in Fig. 2a. Similar results were obtained when we used another anti-TNP–IgG subclasses (IgG1 and IgG2a) for binding experiments (data not shown).

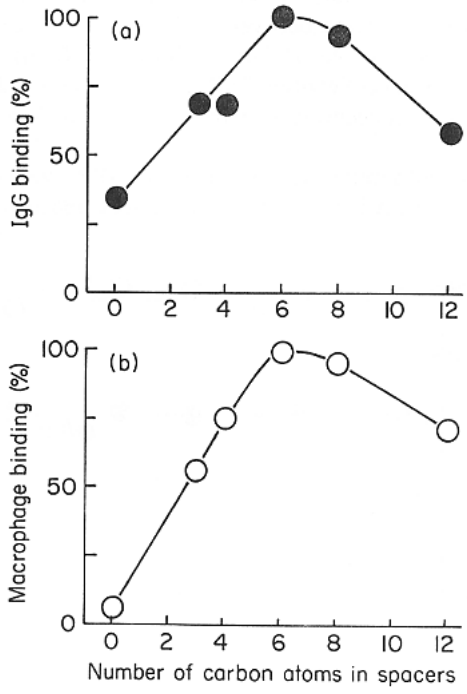



**Figure 2.** Effect of spacer lengths on antibody binding to TNP–lipid haptens and on antibody-dependent macrophage binding to them. (a) Percentages of anti-TNP–IgG2b which was bound to the TNP–lipid haptens (1%) in DMPC liposomes were calculated from tryptophan fluorescence of IgG2b. (b) Antibody-dependent macrophage-binding to the TNP–lipid haptens (1%) in supported planar DMPC monolayers. Percentages of macrophages bound to the IgG2a-coated monolayers were calculated by the procedure described in the Materials and Methods.

These results were well consistent with the previous findings by Dancey *et al.* using dinitrophenylated lipid haptens (Dancey, Isakson & Kinsky, 1979).

### Antibody-dependent binding of macrophages to the TNP–lipid haptens with different length spacers

Macrophages (J774.1 cell line) bound specifically to the IgG-coated lipid monolayers which contained TNP–lipid haptens. Antibody-dependent binding of macrophages to the supported lipid monolayers was dependent on the spacer length as shown in Fig. 2b. The result in Fig. 2b showed that the TNP–lipid hapten containing a very short (TNP–$C_0$-PE or TNP–$C_3$-PE) or a long spacer (TNP–$C_{12}$-PE) was less effective for antibody-dependent macrophage-binding than the lipid hapten containing an intermediate length spacer (TNP–$C_6$-PE). These results indicate that the linker-chain length dependence of the antibody binding is the general phenomena of the lipid membranes from liposomes to the supported-planar membranes.

### Fluorescence energy transfer from pyrene embedded in the bilayers to the TNP–lipid hapten

Figure 3a shows the fluorescence spectra of pyrene-labelled DMPC vesicles. Lipid vesicles with 1-pyrenedodecanoic acid

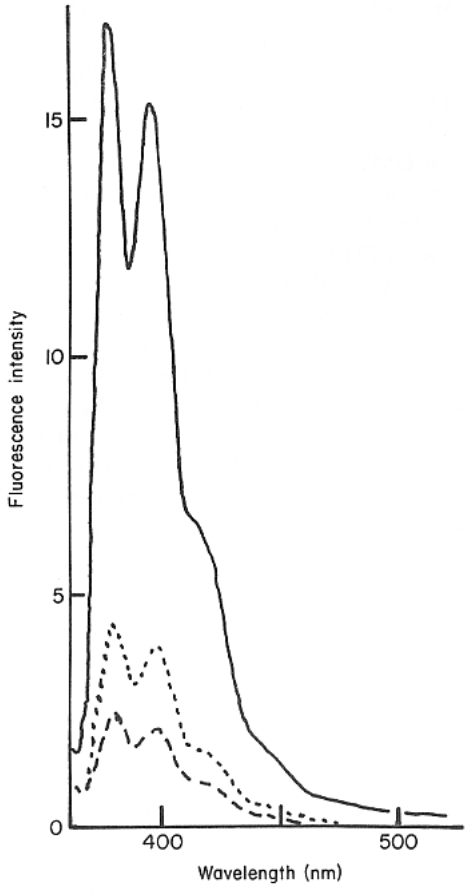



**Figure 3.** Fluorescence spectrum of pyrene dodecanoic acid in DPMC vesicles. DMPC vesicles contained 0·1% pyrenedodecanoic acid. Excited at 340 nm. (———) A fluorescence spectrum without TNP–$C_6$-PE; (· · · · ·) a fluorescence spectrum with 1% TNP–$C_6$-PE; (– – – –) a fluorescence spectrum with 2% TNP–$C_6$-PE.

(0·1%) gave fluorescence peaks around 380–400 nm. This is a typical pattern of a monomer fluorescence of pyrene chromophore (Vanderkooi & Callis, 1974; Galla *et al.*, 1979; Hart, Kimura, & Nakanishi, 1985). A pyrene residue of 1-pyrenedodecanoic acid is known to be situated in the middle of the bilayer membranes. When 1·0% of TNP–$C_6$-PE was present in the pyrene-labelled DMPC vesicles, the fluorescence intensity of a pyrene chromophore in the bilayers decreased drastically as shown in Fig. 3. The fluorescence intensity of pyrene decreased much more when the concentration of TNP–$C_6$-PE increased in the bilayers (Fig. 3). This is due to the fluorescence energy transfer from the pyrene chromophore (donor) in the middle of the bilayers to the TNP groups (acceptors) on the membrane surfaces, because 1-pyrenedodecanoic acid (donor) has emission bands at around 380–400 nm (monomer fluorescence) and TNP groups (acceptors) have a broad absorption band at around these wavelengths. Thus, the excited fluorescence energy is able to transfer from pyrene to TNP.

Substituting acceptor molecules (TNP–$C_6$-PE) for another one (TNP–lipid hapten), fluorescence intensity of 1-pyrenedodecanoic acid decreased similarly to that in the case of TNP–$C_6$-PE (data not shown). These results suggested that the distances between TNP haptenic groups and the pyrene chromophore embedded in the middle of the bilayer membranes were similar values for TNP–lipid haptens with different length spacers.

**Determination of the location of the TNP hapten in the membrane**

So, we determined the vertical distances between TNP haptens and pyrene chromophores in the bilayers using a nanosecond time-dependent fluorescence spectrophotometer. In the absence of TNP–lipids, fluorescence intensity of 1-pyrenedodecanoic acid (0·1%) in DMPC vesicles decayed as shown in Fig. 4a. A life-time of this fluorescence decay was 122 ns at 30°. A life-time of 1-pyrenedodecanoic acid was not changed even if a molecular ratio of pyrene in DMPC vesicles was changed. When TNP–$C_0$-PE (1%) was present in the above vesicles, fluorescence decay of 1-pyrenedodecanoic acid was accelerated as shown in Fig. 4b. An initial-fluorescence decay-rate was 0·0464 $ns^{-1}$ (a life-time of fluorescence decay = 33 ns) (see Table 1). Figure 4c showed fluorescence decay of 1-pyrenedodecanoic acid in the presence of TNP–$C_6$-PE (1%). Initial fluorescence decay-rates was 0·0445 $ns^{-1}$ for DMPC vesicles containing TNP–$C_6$-PE (1%). In initial fluorescence decay-rates were almost independent on the linker chain length of the TNP–lipid haptens as shown in Fig. 4 and Table 1.

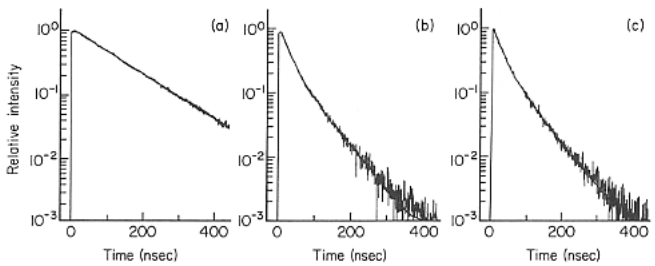



**Figure 4.** Nanosecond time-dependent fluorescence decay of pyrene dodecanoic acid in DMPC vesicles. DMPC vesicles contained 0·1% pyrenedodecanoic acid. (a) A fluorescence decay of pyrene-labelled DMPC without TNP–lipids. (b) A fluorescence decay of pyrene-labelled DMPC with 1% TNP–$C_0$-PE. (c) A fluorescence decay of pyrene-labelled DMPC with 1% TNP–$C_6$-PE.

**Table 1.** Experimental values of nanosecond fluorescence measurements of 1-pyrenedodecanoic acid in the membranes and the location of the membrane bound TNP haptens

| | Initial fluorescence decay rate,* $k_d$ | | Distance† | |
|---|---|---|---|---|
| | (DMPC) ($ns^{-1}$) | (DPPC) ($ns^{-1}$) | (DMPC) (Å) | (DPPC) (Å) |
| TNP–$C_0$-PE | 0·0464 | 0·0215 | 10·2 | 13·7 |
| TNP–$C_3$-PE | 0·0415 | 0·0227 | 10·5 | 13·5 |
| TNP–$C_4$-PE | 0·0427 | 0·0233 | 10·4 | 13·4 |
| TNP–$C_6$-PE | 0·0445 | 0·0212 | 10·3 | 13·8 |
| TNP–$C_8$-PE | 0·0434 | 0·0208 | 10·4 | 13·9 |
| TNP–$C_{12}$-PE | 0·0440 | 0·0244 | 10·4 | 13·2 |

*The initial fluorescence decay rate ($k_d$) was estimated by fitting an observed fluorescence decay with a double-exponential function; $a_1\exp(-t/\tau_1)+a_2\exp(-t/\tau_2)$, $a_1+a_2=1$ and setting $k_d=a_1/\tau_1+a_2/\tau_2$.

†This value means a vertical distance between TNP groups (acceptors) and the pyrene group (donor) in the middle of the bilayer membranes. We obtained that $R_0$ values in the equation (1) were 25·8 Å and 27·1 Å for the DMPC and DPPC membranes, respectively.

Using the equation (1), we calculated vertical distances of TNP haptens (acceptors) from the pyrene fluorophore (donor) in the middle of DMPC bilayers as described in the Materials and Methods. These results are shown in Fig. 5. In DMPC vesicles, which are fluid at 30°, the vertical distances between TNP and pyrene were between 10·2 and 10·5 Å for different length spacers (Table 1). In addition we found that the calculated distances between TNP and pyrene were independent of the acceptor concentration in the membrane (0·2–2·0% TNP–$C_n$-PE).

We studied similar experiments using DPPC vesicles, which are solid at 30°. A life-time of pyrenedodecanoic acid in DPPC

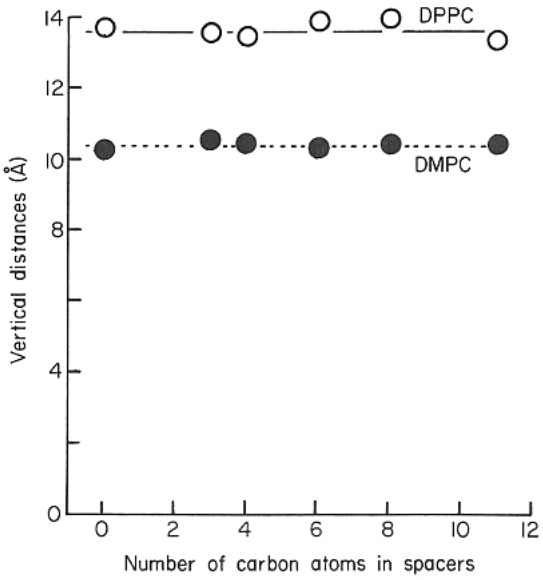



**Figure 5.** Vertical distances between TNP groups and a pyrene fluorophore which was embedded in the middle of the lipid bilayers. Closed circles are distances in the DMPC membranes. Open circles are distances in the DPPC membranes.

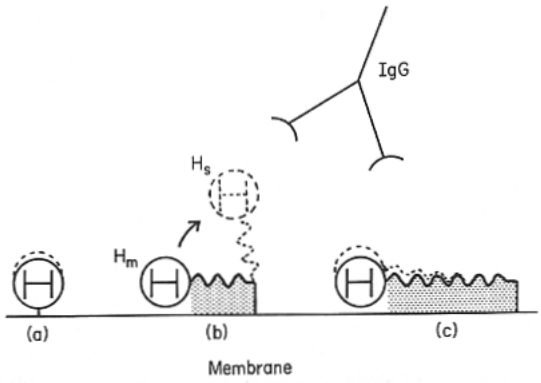



**Figure 6.** Schematic representation of the TNP haptens with (a) a shorter, (b) an intermediate and (c) a longer spacer in the state of $H_m$ of the equation (2). TNP haptens locate at the similar vertical position on the membrane surfaces even if they have different length spacers. However, a longer spacer group (in TNP–$C_{12}$-PE) interacts strongly with the bilayer surfaces and TNP groups unavailable for antibody binding. Haptens with the intermediate length spacers (in TNP–$C_6$-PE) are able to be easily transferred from the bilayer surfaces (state $H_m$) to the aqueous solution (state $H_s$). In the state of $H_s$, TNP haptens with the intermediate length spacer (in TNP–$C_6$-PE) are available for antibody binding without steric constraints from the membrane surfaces. Haptens with shorter spacers (TNP–$C_0$-PE or TNP–$C_3$-PE) are less effective for antibody binding due to steric constraints.

vesicles in the absence of TNP–lipid haptens was 167 ns. When the TNP–lipid was present in the vesicles, fluorescence decay of 1-pyrenedodecanoic acid was accelerated. Initial fluorescence decay rates of pyrene in the presence of TNP–lipid haptens are summarized in Table 1. The vertical distances from pyrene to TNP were between 13·2 and 13·9 Å as shown in Fig. 5. The vertical distances between TNP and pyrene were about 3 Å longer in the solid membranes (DPPC vesicles) than in the fluid membranes (DMPC vesicles). However, they were almost independent of the linker chain lengths for both solid and fluid membranes. This indicates that the TNP–lipid haptens with different length spacers must be located at almost the same vertical position on the surfaces of DMPC and DPPC membranes.

## DISCUSSION

The present results showed that the vertical distances between TNP haptenic groups and a pyrene chromophore in the bilayers were 10·2–10·5 Å for DMPC membranes and 13·2–13·9 Å for DPPC membranes. The vertical distances were almost independent of spacer lengths. This indicated that the haptenic groups in lipid haptens with the longer spacers (TNP–$C_{12}$-PE) are neither extended away from the bilayer membranes nor are they become buried within the bilayers. If so, why do antibody molecules bind more strongly to the lipid hapten with the intermediate spacer length (TNP–$C_6$-PE) as shown in Fig. 2 or the references (Dancey, Isakson & Kinsky, 1979; Alving & Richards, 1983)?

The results are most simply accounted for if, in the absence of antibody, there exists an equilibrium between two states for hapten residues, $H_m$ and $H_s$ (Balakrishnam, Mehdi & McConnell, 1977; Stanton *et al.*, 1984):

$$H_m \rightleftharpoons H_s. \qquad (2)$$

Here $H_m$ represents a physical state of the TNP group which interacts with bilayer membranes and is unavailable for antibody binding. $H_s$ represents a physical state of the TNP group which is exposed in aqueous solution and available for antibody binding. In addition, most TNP groups which are covalently bonded phosphatidylethanolamine are considered to exist in the state of $H_m$.

Schematic representation of the TNP groups in the state of the membrane bound ($H_m$) is shown in Fig. 6. In the $H_m$ state, the hydrophobic interaction between a longer spacer and lipid bilayers must be much stronger than that of a shorter spacer or an intermediate spacer. If so, even if TNP residues in TNP–$C_{12}$-PE and TNP–$C_6$-PE locate at the same vertical position on the membrane surfaces, TNP groups in TNP–$C_{12}$-PE are more difficult to transfer from the $H_m$ state (bound to the membranes) to the $H_s$ state (free in solution) than TNP groups in TNP–$C_6$-PE. That is, TNP groups with longer spacers become unavailable for antibody binding.

This explanation was partly supported by the following experiments. That is, we measured the binding affinity of model compounds of TNP–spacers (without phospholipids) to the bilayer membranes. TNP–*N*-propylic acid (TNP-$C_3$), TNP–*N*-caproylic acid (TNP–$C_6$) and TNP–*N*-laurylic acid (TNP–$C_{12}$) were incubated with preformed DMPC multilamellar liposomes at 30° for 5 min. After that, we measured the amount of TNP spacers bound to liposomes. TNP–*N*-laurylic acid (TNP–$C_{12}$) bound much stronger to the DMPC membranes than TNP–*N*-caproylic acid ( TNP–$C_6$) and TNP–N-propylic acid (TNP–$C_3$) did.

In the case of shorter spacers (TNP–$C_0$-PE or TNP–$C_3$-PE) the hydrophobic interaction between spacers and the bilayers is weaker. However, the TNP groups which are covelently bonded to PE are not exposed completely into an aqueous solution, because of their shorter length spacers. Or, there is a substantial side group effect with anti-TNP–IgGs due to PE conjugation in the immunogen. Thus, TNP haptens with very short spacers (TNP–$C_0$-PE and TNP–$C_3$-PE) are less effective for antibody binding due to steric constraints from membrane surfaces (or a substantial side group effect) than that of the intermediate length spacer (TNP–$C_6$-PE).

We showed in the present experiments that the vertical distances between a pyrene and TNP groups were 3 Å longer in the solid DPPC membranes than in the fluid DMPC membranes. This does not mean that TNP groups in the DPPC membranes are more exposed in an aqueous solution than those in the DMPC membranes. It is more plausible that increased flexibility of acyl-chains in the DMPC membranes shortens the vertical distance between the pyrene and TNP groups.

In conclusion, the present results showed that the TNP groups of lipid haptens located at the similar vertical position on the membrane surfaces even if they have different length spacers. The results suggested that the affinity of the spacer groups to the bilayer surfaces can modulate the binding affinity of antibody to lipid hapten on the membrane surfaces.

## ACKNOWLEDGMENTS

We would like to thank Professor Akira Ikegami and Dr Tsutomu Kouyama for their helpful discussion.

## REFERENCES

ALVING C.R. & RICHARDS R.L. (1983) Immunologic aspect of liposomes. In: *Liposomes* (ed. M.J. Ostro), p. 209. Marcel Dekker, NY.

BALAKRISHNAN K., MEHDI S.Q. & MCCONNELL H.M. (1982) Availability of dinitrophenylated lipid haptens for specific antibody binding depends on the physical properties of host bilayer membranes. *J. Biol. Chem.* **257,** 6434.

BATZRI S. & KORN E.D. (1973) Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta*, **298,** 1015.

BRÛLET P. & MCCONNELL H.M. (1977) Structural and dynamical aspects of membrane immunochemistry using model membranes. *Biochemistry*, **16,** 1209.

DANCEY G.F., ISAKSON P.C. & KINSKY S.C. (1979) Immunogenicity of liposomal model membranes sensitized with dinitrophenylated-phosphatidylethanolamine derivatives containing different length spacers. *J. Immunol.* **122,** 638.

FORSTER T. (1965) Delocalized excitation and excitation transfer. In: *Modern Quantum Chemistry* (ed. O. Sinanoglu), p. 93. Academic Press, New York.

GALLA H.J., HARTMANN, W., THEILEN U. & SACKMANN E. (1979) On two-dimensional passive random walk in lipid bilayers and fluid pathways in biomembranes. *J. Membr. Biol.* **48,** 215.

HAFEMAN D.G., TSCHARNER V. & MCCONNELL H.M. (1981) Specific antibody-dependent interactions between macrophages and lipid haptens in planar lipid monolayers. *Proc. natl. Acad. Sci. U.S.A.* **78,** 4552.

HART M.J., KIMURA K. & NAKANISHI M. (1985) Selected positions of acyl chains are affected differently by antibody binding which results in decreased membrane fluidity. *FEBS Lett.* **190,** 249.

HO R.J.Y. & HUANG L. (1985) Interactions of antigen-sensitized liposomes with immobilized antibody: A homogeneous solid-phase immuno-liposome assay. *J. Immunol.* **134,** 4035.

KIMURA K. & NAKANISHI M. (1985) Subclass-specific antibody-dependent binding of macrophages to supported planar lipid monolayers membranes. *FEBS Lett.* **187,** 69.

KIMURA K., NAKANISHI M., UEDA M., UENO J., NARIUCHI N., FURUKAWA S. & YASUDA T. (1986) The effect of immunoglobulin G1 structure on macrophage binding to supported planar lipid monolayers. *Immunology*, **59,** 235.

KINOSITA K., JR., KATAOKAR R., KIMURA K., GOTOH O. & IKEGAMI, A. (1981) Dynamic structure of biological membranes as probed by 1,6-diphenyl-1,3,5-hexatriene: a nanosecond fluorescence depolarization study. *Biochemistry*, **20,** 4270.

KLEINFIELD A.M. & LUKACOVIC M.F. (1985) Tryptophan imaging of membrane proteins. *Biochemistry*, **24,** 1883.

KOMETANI T., KINOSITA K. J., FURUNO T., KOUYAMA T. & IKEGAMI A. (1987) Transmembrane location of retinal in purple membrane. *Biophys. J.* **52,** 509.

KREMER J.M.H., ESKER M.W.J., PATHMAMANOHARAN C. & WIERSEMA P.H. (1977) Vesicles of variable diameter prepared by a modified injection method. *Biochemistry*, **16,** 3932.

OKADA N., YASUDA T., TSUMITA T. & OKADA H. (1982) Activation of the alternative complement pathway of guinea-pig by liposomes incorporated with trinitrophenylated phosphatidylethanolamine. *Immunology*, **45,** 115.

PHILIP Y. (1987) *The Membrane of Cells*. Academic Press. New York.

SIX H.R., UEMURA K., & KINSKY S.C. (1973) Effect of Immunoglobulin class and affinity on the initiation of complement-dependent damage to liposomal model membranes sensitized with dinitrophenylated phospholipids. *Biochemistry*, **12,** 4003.

SIX H.R., YOUNG W.W., UEMURA K., & KINSKY S.C. (1974) Effect of antibody-complement on multiple vs single compartment liposomes. Application of a fluorometric assay for following changes in liposomal permeability. *Biochemistry*, **13,** 4050.

STANTON S.G., KANTOR A.B., PETROSSIAN A., & OWICKI J.C. (1984) Location and dynamics of a membrane-bound fluorescent hapten. A spectroscopic study. *Biochim. Biophys. Acta*, **776,** 228.

STRYER L. (1978) Fluorescence energy transfer as a spectroscopic ruler. *Ann. Rev. Biochem.* **47,** 819.

THOMAS D.D., CARLSEN W.F., & STRYER L. (1978) Fluorescence energy transfer in the rapid diffusion. *Proc. natl. Acad. Sci. U.S.A.* **75,** 5746.

VANDERKOOI J.M., & CALLIS J.B. (1974) Pyrene. A probe of lateral diffusion in the hydrophobic region of membranes. *Biochemistry*, **13,** 4000.