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Lanthanum and Some Other Cation-Induced Changes in Fluidity of Synaptosomal Membrane Studied with Nitroxide Stearate Spin Labels

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Summary. Using nitroxide fatty acid spin labels, the effects of some cations such as La^{3+}, Cd^{2+} and Hg^{2+} on synaptosomal membranes were studied by observing changes in their ESR spectra. The labels were incorporated almost instantaneously into synaptosomes isolated from rat brain cortex. ESR spectra of the spin-labeled synaptosomes were significantly broadened immediately upon adding La^{3+}, Ce^{3+}, Cd^{2+} or Hg^{2+} but hardly affected by Ca^{2+}, Sr^{2+} and Ba^{2+}. The magnitude of the change in the separation of the outer two peaks in ESR spectra ($2T'_{\parallel}$) depends on the number (n) of methylene units between the polar head group and the spin-label (nitroxide) group; that is, it increases with decreasing n. Among these ions, the effect of La^{3+} was the greatest and appeared to be in parallel with the amount of La^{3+} bound with the synaptosomes. On the other hand, K^+, Rb^+ or Li^+ causes hardly any significant changes.

It has been well documented that, in the process of excitation or excitation-secretion coupling, Ca^{2+} played an essential role, while Mg^{2+}, Mn^{2+} and La^{3+} antagonized such an action of Ca^{2+} (*cf.* Dodge & Rahamimoff, 1967; Hagiwara & Takahashi, 1967; Kajimoto & Pirpeker, 1972). Our previous studies on synaptosomes also demonstrated that synaptosomal membranes bound Ca^{2+}, and the Ca-binding was inhibited by high external K^+ (Kamino, Uyesaka & Inouye, 1974), by Mg^{2+} and other alkali earth metal ions as well as by some heavy metal ions such as Mn^{2+} and La^{3+} which were actually bound with synaptosomal Ca-binding sites (Kamino, Inouye, Ogawa, Uyesaka & Inouye, 1975*a*; Kamino, Uyesaka, Ogawa & Inouye, 1975*b*). It was suggested in these studies that certain structural changes in synaptosomal membranes occurred in Ca-binding and/or in inhibition by some ions. Systematic studies on the conformation effects of these ions on synaptosomal membranes are, however, lacking

and the molecular mechanism underlying the ion-participated physiological phenomena still remains unclear.

The spin-label technique, applied to problems of biological membranes can, in principle at least, give information on the molecular architecture and the dynamic behavior of the membranes (McConnell & McFarland, 1970; Jost, Waggoner & Griffith, 1971; Keith, Sharnoff & Cohn, 1973). Applying this technique, therefore, we attempted ESR studies on the interactions between synaptosomal membranes and some cations. Results obtained with nitroxide fatty acid spin labels are described herein.

Materials and Methods

Preparation of Synaptosomes from Rat Brain Cortex

Essentially the same methods as those used in our work (Kamino, Inouye & Inouye, 1973) were employed. Pooled cortical tissues (usually two or three brains/one preparation) were homogenized in 0.32M sucrose by a Teflon glass homogenizer of Potter-Elvejem type.

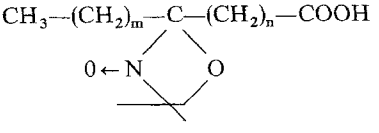
Using a Hitachi preparative ultracentrifuge (Type HU 40 P or HU 65 P), the synaptosomes were prepared by sucrose density gradient centrifugation according to the procedure of Gray and Whittaker (1962), with slight modification. The microsomal supernatant was separated by centrifugation at 12,000 × *g* for 20 min and the crude mitochondrial fraction thus obtained was subjected to the density gradient centrifugation at 64,000 × *g* for 60 min. The material at the 0.8 to 1.2M interface was used as the synaptosomal fraction after usually two washings with resuspension and centrifugation (12,000 × *g* for 20 min) in buffered 0.32M sucrose.

Preparation of myelin from rat brain medulla was made according to the procedure of Autilio, Norton and Terry (1964).

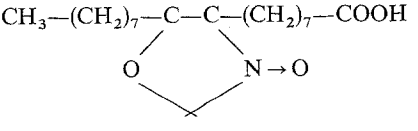
The final pellets were once washed in suspension and centrifugation (synaptosomes: 12,000 × *g* for 20 min; microsomes: 78,000 × *g* for 120 min; myelin: 40,000 × *g* for 10 min) in buffered 170mM NaCl solution (pH 7.3, 15 mM Tris-Cl buffer). Finally, the synaptosomal, microsomal and myelin suspension in 170mM NaCl solution was submitted to ESR measurements.

Spin Labels and ESR Measurements

As spin labels we have used N-oxyl-4′, 4-dimethyl-oxazolidine derivatives of stearic acid with the general formula, $I(m, n)$:



as well as the derivative of oleic acid,



its oxazolidine ring is co-planar with that of the alkyl pleat. In the present study, $I(12, 3)$, $I(5, 10)$ and $I(1, 14)$ were employed and denoted as 5-NS, 12-NS and 16-NS, respectively, by the position of the nitroxide group from the polar head group, with the oleic acid derivative as 9,10-NS. The probe 16-NS was purchased from Synvar; the other three probes were synthesized and supplies from the Laboratory of Professor S. Ohnishi, Department of Biophysics, Faculty of Sciences, Kyoto University.

The spin labels were incorporated into synaptosomal membranes; as described by Seelig and Hasselbach (1971), the labels were first dissolved in ethanol, the solvent was evaporated in a small round-bottomed flask, and then a suspension of synaptosomes in 170 mM NaCl was added. With this procedure, the labels were found to be incorporated almost instantaneously into the synaptosomes, despite their low solubility in 170 mM NaCl solution. The concentration of the synaptosomal suspensions applied was usually $3 \sim 5$ mg protein/ml; that of the spin labels approximately 10 μg/ml.

The suspensions thus treated were subjected to centrifugation at $12{,}000 \times g$ for 20 min and the pellets were put into the cylindrical quartz tube for ESR measurements. All ESR spectra were measured at 22 °C by a JEOL JES-3 BS-X spectrometer.

Results

Characteristics of ESR Spectra of Spin-Labeled Synaptosomes

For a correct interpretation of the ESR spectra of the spin-labeled fatty acids, when in combination with synaptosomes, we first made measurements on the synaptosomes labeled with a probe such as 5-NS, 9, 10-NS, 12-NS or 16-NS.

Fig. 1 contains a series of ESR spectra which were obtained with synaptosomes probed with 5-NS, 9, 10-NS, 12-NS or 16-NS. Their spectra are quite similar to those of labeled lipid bilayer reported by Seelig (1970, 1971) and Hubbell and McConnell (1971), a finding which indicates that the stearic acid labels are incorporated chiefly, if not solely, into the lipid bilayer of synaptosomal membrane. Such incorporation was also found on brain microsomes and myelin fragments, both preparations provided the spectra nearly identical with those presented in Fig. 1. As shown in Fig. 1, the broadening in the ESR spectrum, *viz.*, increasing in the $2T'_{\parallel}$, appeared with decreasing the bond number n of methylene units between the polar head group and the spin-label group. The relationship between the $2T'_{\parallel}$ observed and n of spin labels used is shown in Fig. 2*A*. The Figure shows that the $2T'_{\parallel}$ of synaptosomes labeled with 5-NS is nearly identical with that reported on other biological membranes such as sarcoplasmic reticulum membranes from rabbit skeletal muscle (53.8 G, Seelig & Hasselbach, 1971) and erythrocyte membranes (55.8 G for human, Simpkins, Panko & Tay, 1971; 56.8 G for dog, Hubbell & McConnell, 1969), while the value is greater than that in phospholipid dispersion (51.0 G, Seelig & Hasselbach, 1971).

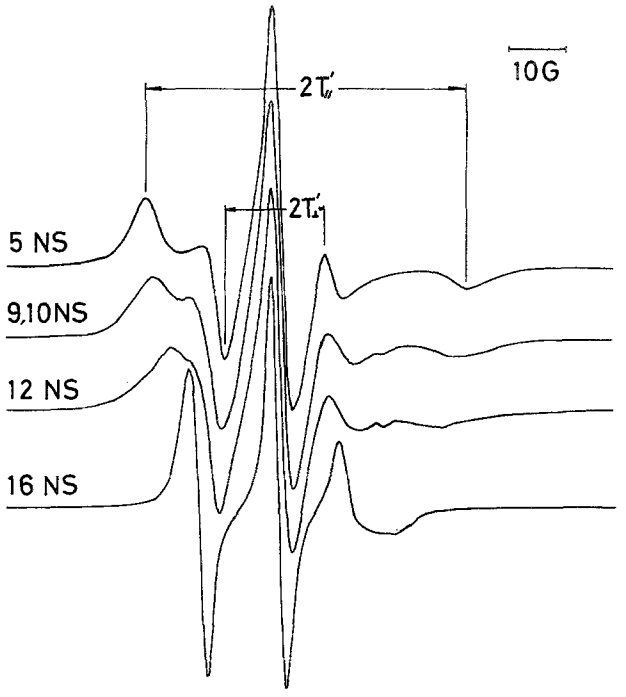


Fig. 1. ESR spectra of various nitroxide stearate spin labels incorporated into synaptosomal pellets of suspensions in 170 mM NaCl solution (Tris-Cl buffer 15 mM, pH 7.3). Temp. 22 °C

Each spectrum corresponds to a different state of motion of spin label. Hence the order parameter S for each spectrum was calculated. All the spectra can be interpreted with an axially symmetrical spin Hamiltonian. The parallel ($T'_{\parallel}$) and perpendicular ($T'_{\perp}$) principal value of the hyperfine tensor was estimated from the spectra. The order parameter S was calculated using the relation:

$$S=(T'_{\parallel}-T'_{\perp})/(T_{zz}-T_{xx})(a/a'),$$

where a' is the isotropic nitrogen hyperfine coupling constant and this can be calculated from the values of $T'_{\parallel}$ and $T'_{\perp}$ as $a'=\frac{1}{3}(2T'_{\parallel}+T'_{\perp})$. T_{zz}, T_{xx}, and a were obtained from the data of Hubbell and McConnell (1971). The values of $T'_{\parallel}$, $T'_{\perp}$ and a' for spin labels used for calculation are summarized in Table 1.

In the spectra of 5-NS, 9, 10-NS and 12-NS, the spin label motion is probably anisotropic ($S>0.3$). (S is not, strictly speaking, in the correct form for use with 9, 10-NS.) In the spectra of 16-NS, an exact determination of S is difficult, but it can be estimated that $S<0.3$. This value shows that the spin label motion of 16-NS is almost isotropic.

Table 1. Parameters of ESR spectra used for computation of the order parameter of nitroxide stearate spin labels in synaptosomes suspended in 170 mM NaCl (pH 7.3, Tris-Cl buffer 15 mM) at 22 °C

Labels	$T'_{\parallel}$(G)	$T'_{\perp}$(G)	a'(G)
5-NS	28.1	8.7	15.1
9, 10-NS	26.6	9.1	14.9
12-NS	23.4	9.4	14.0
16-NS	17.9	11.3	13.5

Fig. 2*B* shows a plot of the logarithm of S against the bond number n. Here, the value of S decreases with increasing n, but no linear relationship was obtained between S and n; in the range of $n<7\sim8$, the decrease in S is small, but becomes larger abruptly by increasing n from 10 to 14. Such a behavior of S with varying n appears to be generally observed on biological membranes such as sarcoplasmic reticulum membranes (Seelig & Hasselbach, 1971), brain microsomes and myelin fragments (Fig. 2*B*).

Effects of Cations on the $2T'_{\parallel}$ or the Order Parameters

Effects of various cations such as Ca^{2+}, Cd^{2+} and La^{3+} on the $2T'_{\parallel}$ or S in spin-labeled synaptosomes were studied in connection with structural changes in the membrane or Ca-binding of synaptosomes (Kamino *et al.*, 1974, 1975*a*, *b*).

Effects of La^{3+} and Ce^{3+}. La^{3+} has been reported to strongly inhibit Ca-binding of synaptosomes (Kamino *et al.*, 1975*b*), endoplasmic reticulum (Krasnow, 1972), mitochondria (Lehninger & Carafoli, 1971), or sarcolemma (Kajimoto & Pirpeker, 1972), while it was known that Ce^{3+} inhibits the effects of Ca^{2+} in neuromuscular junction (Hubbard, 1973). We also showed that Ce^{3+} inhibited Ca^{2+} binding of synaptosomes (Kamino *et al.*, 1975*b*). Incubating spin-labeled synaptosomes in 170 mM NaCl media (pH 7.3, Tris-Cl buffer 15 mM) containing $LaCl_3$ or $CeCl_3$ of various concentrations at 25 °C for 10 min, the effects of these ions were studied. As clearly seen in Fig. 3, $2T'_{\parallel}$ of the spectrum of 5-NS label significantly increases in the presence of $LaCl_3$ (or $CeCl_3$) of 3 mM; the $2T'_{\parallel}$ changes from about 56.0 G to about 62.5 G, i.e. $\Delta 2T'_{\parallel}=6.5$ G. As shown in Table 2, the magnitude of the changes in $2T'_{\parallel}$ or in S decreases with increasing n, the magnitude being greater in synaptosomes probed with 5-NS.

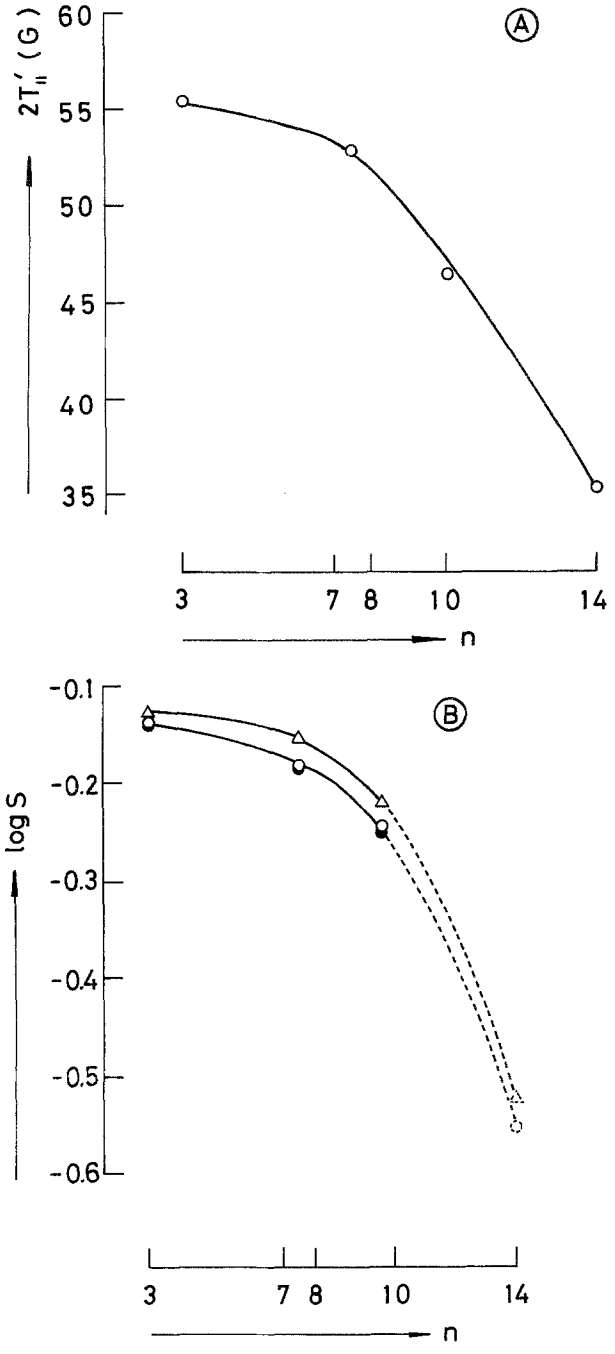


Fig. 2. (*A*) Relationship between $2T_{\parallel}'$ and the number of CH_2-groups between nitroxide and COOH groups n of nitroxide stearate spin labels in synaptosomes in 170 mM NaCl solution (pH 7.3, Tris-Cl buffer 15 mM). (*B*) Relationship between the order parameter S and n of nitroxide stearate spin labels in synaptosomes (○), microsomes (●) and myelin (△) in 170 mM NaCl solution (pH 7.3, Tris-Cl buffer 15 mM). Temp. 22 °C

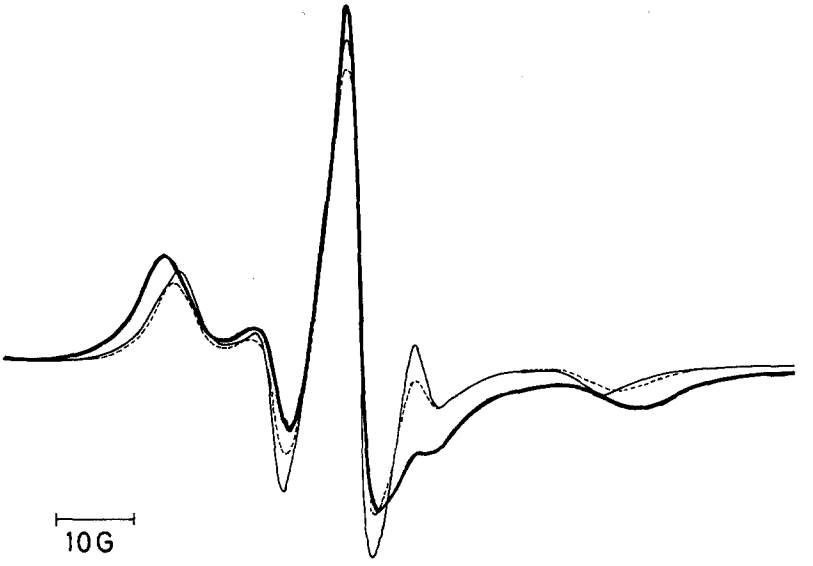


Fig. 3. ESR spectra of 5-NS in synaptosomes in the presence of La^{3+}, Ce^{3+} or Cd^{2+}. The traces by fine lines, the control (170 mM NaCl, 15 mM Tris-Cl buffer of pH 7.3); thick lines, La^{3+} (3 mM) or Ce^{3+} (3 mM) added; broken lines, Cd^{2+} (3 mM). Temp. 22 °C

Table 2. Effects of some heavy metal ions on $2T'_{\parallel}$ of nitroxide stearate spin labels in synaptosomes, microsomes and myelin. Temp. 22 °C

Labels	Cations	Synaptosomes ($n=4$)	Microsomes ($n=3$)	Myelin ($n=3$)
		$2T'_{\parallel}$(G)	$2T'_{\parallel}$(G)	$2T'_{\parallel}$(G)
5-NS	control	56.1±0.08	56.0±0.06	59.3±0.04
	La^{3+} (3 mM)	62.9±0.10	61.7±0.09	62.8±0.06
	Ce^{3+} (3 mM)	62.9±0.08	–	–
	Cd^{2+} (3 mM)	58.3±0.06	–	60.7±0.05
	Hg^{2+} (3 mM)	57.4±0.05	–	61.0±0.06
12-NS	control	46.7±0.06	46.6±0.03	51.4±0.06
	La^{3+}	47.9±0.08	47.6±0.05	52.5±0.06

The values presented are the mean±SE.

La^{3+}-induced change in $2T'_{\parallel}$ or order parameter is dependent on the concentration of La^{3+} added. Fig. 4 shows the relationship between the $2T'_{\parallel}$ of synaptosomes probed with 5-NS and the concentration of La^{3+} added to the 170 mM NaCl solution. The $2T'_{\parallel}$ increased hyperbolically as the concentration of La^{3+} increased, leveling off at around 4.0 mM of La^{3+}. We suggested in our previous report that the binding of La^{3+} with synaptosomal Ca^{2+}-binding sites was the adsorption of Langmuir type. As shown in Fig. 5, a linear correlation of the slope $\simeq 1$ was obtained

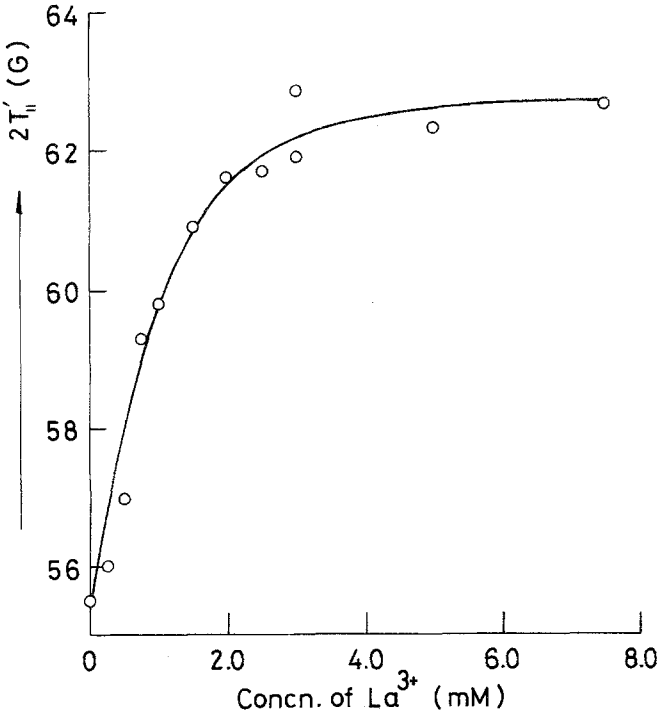


Fig. 4. Relationship between $2T_{\parallel}'$ and the concentration of La^{3+} added for 5-NS in synaptosomes

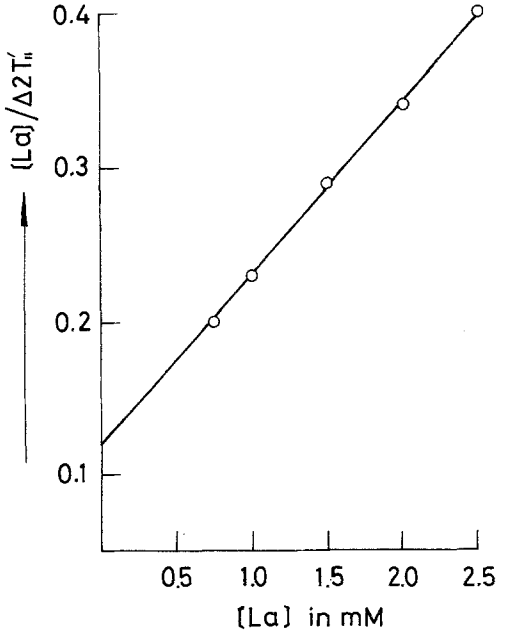


Fig. 5. The plot of Langmuir type constructed from Fig. 4. *Abscissa:* the concentration in mM of La^{3+} added; *Ordinate:* La^{3+} concentration in mM per $\Delta 2T_{\parallel}'$ in G

between $[La^{3+}]/\Delta 2T'_{\parallel}$ and $[La^{3+}]$, where $[La^{3+}]$ is the concentration of added La^{3+}, $\Delta 2T'_{\parallel}$ an increase in $2T'_{\parallel}$. The equilibrium constant estimated from this plot is about 1.2 mM, a value 5 times greater than the value for the binding of La^{3+} with Ca^{2+}-binding sites. These results suggest that La^{3+}-induced increase in $2T'_{\parallel}$ originates from binding of La^{3+} with the surface of synaptosomal membrane, but not from its binding with Ca^{2+}-binding sites.

The results obtained with Ce^{3+} were quite similar to those with La^{3+} (Fig. 3). The inhibitory effect of Ce^{3+} on Ca^{2+}-binding of synaptosomes was demonstrated to be quite similar to that of La^{3+}. These results show that Ce^{3+} also binds to the surface of the membrane and introduces the changes in the ESR spectra just as La^{3+}.

In the presence of La^{3+} (3 mM), $2T'_{\parallel}$ of the spectra of spin-labeled microsomes as well as myelin fragments also increased as that of synaptosomes. As summarized in Table 2, the magnitude of the change in $2T'_{\parallel}$ or in S decreases with increasing n.

Effects of some Divalent Heavy Metal Ions. The effect of Cd^{2+} and Hg^{2+} on the ESR spectra of the spin-labeled synaptosomes are also included in Fig. 3 and Table 2. As seen in the Figures, 3 mM of Cd^{2+} or Hg^{2+} results in broadening of the spectra, especially an increase in $2T'_{\parallel}$ and also an increase in S. Such effects by Cd^{2+} and Hg^{2+} are quite similar to but less than those by La^{3+} and Ce^{2+}; $\Delta 2T'_{\parallel}$ for the 5-NS is about 2 G with Cd^{2+} and 1.5 G with Hg^{2+}. Here it should be noted, however, that $HgCl_2$ was not fully ionized under the present experimental conditions, the concentration of Hg^{2+} being significantly lower than 3 mM. Such an effect of Cd^{2+} and Hg^{2+} of increasing $2T'_{\parallel}$ was also observed on myelin fragments labeled with 5-NS (Table 2).

On the other hand, 3 mM of Co^{2+} or Ni^{2+}, which bind not only with Ca-binding sites but also with non-Ca-binding sites of synaptosomes (Kamino *et al.*, 1975*a*), causes only a slight broadening of the spectra ($\Delta 2T'_{\parallel} < 1$ G). Hardly any alteration of the ESR spectra was observed, as shown below, when synaptosomal Ca-binding sites were occupied by Ca^{2+} and other alkali earth metal ions. Thus, changes in the spectra produced by Cd^{2+} and Hg^{2+} do not appear to be the result of conformational changes related to Ca-binding sites, but rather due to their binding to the membrane surface quite similar to that of La^{3+}.

Effect of Alkali Earth Metal Ions. In the presence of Ca^{2+}, Mg^{2+}, Sr^{2+} or Ba^{2+} (up to 6 mM), no significant change in ESR spectra of labeled synaptosomes was observed. The alkali earth metal ions other than Ca^{2+} also bind with synaptosomal Ca-binding sites, resulting in a competitive

inhibition (Kamino *et al.*, 1975*b*). Consequently, it is said that ESR studies with nitroxide stearate labels cannot detect conformational changes due to a combination of these ions with synaptosomal Ca-binding sites. Indeed, ruthenium red (up to 50 μM), a dye which is known to be a powerful inhibitor of Ca^{2+}-binding and firmly bound with synaptosomal Ca-binding sites (Kamino *et al.*, 1975*a*), did not cause any change in ESR spectra of synaptosomes labeled with nitroxide stearate, irrrespective of presence or absence of Ca^{2+}.

Effects of Alkali Metal Ions. Based on our findings (Kamino *et al.*, 1973, 1974), high external K^+ (or Rb^+) not only causes swelling of synaptosomes but also shows a specific noncompetitive inhibition of synaptosomal Ca-binding, whereas Li^+ never shows these effects on synaptosomal membranes. In the present study, therefore, we examined the effect of these ions on the ESR spectra of spin-labeled synaptosomes.

Not surprisingly the ESR spectra of synaptosomes were hardly affected by 170 mM LiCl. In 170 mM KCl or RbCl, a slight decrease in the $2T'_{\parallel}$ (about 1 G for K^+ and about 0.5 G for Rb^+) was observed only when spin-labeled with 9, 10-NS or 12-NS, but never with 5-NS or 16-NS. At the present stage of investigations, however, such minute decrease in $2T'_{\parallel}$ cannot be regarded as statistically significant.

Discussion

The results presented above showed that the trivalent cations La^{3+} and Ce^{3+}, as well as some divalent heavy metal ions such as Cd^{2+} and Hg^{2+} caused a significant alteration of the ESR spectra of synaptosomes spin-labeled with the nitroxide stearate probes, whereas the alkali earth metal ions having a specific affinity for so-called "Ca-binding sites" hardly affected the spectra.

Seelig (1970) demonstrated experimentally that spin-labeled stearic acid probes incorporated into the bilayer structure of lipids. The ESR spectra shown in Fig. 1 suggested that the region of synaptosomal membrane labeled with the nitroxide probes was mainly, if not solely, the lipid bilayer phase. In view of the fact that a Ca-binding protein was isolated from the brain of rat (Wolff, Huebner & Siegel, 1972), which had characteristics of binding quite similar to that of synaptosomes, the synaptosomal Ca-binding sites are undoubtedly of a protein nature. Indeed, we succeeded recently in isolating some sorts of Ca-binding proteins from rat synaptosomes, which showed Ca-binding activity similar to that of synaptosomes studied hitherto in our laboratory (Ogawa, Kamino,

Uyesaka, Inouye, *in preparation*). It seems not surprising, therefore, that binding of an ion with the Ca-binding sites scarcely causes any significant effect on the ESR spectra of labeled synaptosomes, when the interaction of Ca-binding sites with the membrane lipid bilayer phase is not so strong. Thus, it might be said that La^{3+} and other ions, despite their high affinity for Ca-binding sites, result in the broadening of the ESR spectra by their binding with non-Ca-binding sites of synaptosomes. Of course, the "non-Ca-binding sites" included, partly at least, the regions of lipid bilayer in the synaptosomal membranes.

The broadening or the increase in $2T'_{\parallel}$ observed in the ESR spectra of the nitroxide fatty acid probes probably arises, as described by Ito and Ohnishi (1974), from intramolecular dipolar broadening due to reduced mobility of the lipid alkyl chain and such would reflect a decrease in so-called fluidity of the molecules surrounding the nitroxide. From such a point of view, the relationship between the order parameter (S) and the bond number (n) (Fig. 4*B*) can be interpreted as an indication of a larger fluidity in deeper regions of the synaptosomal membrane phase, a finding which seems, as Hubbell and McConnell (1971) pointed out, to have a common nature in both biological and artificial membranes. In the artificial lipid membranes, a linear correlation is usually observed between S and n (Seelig, 1970; Hubbell & McConnell, 1971). The synaptosomes, microsomes, and myelin show, however, a marked deviation from the linear correlation just as the sarcoplasmic reticulum studied by Seelig and Hasselbach (1971). Such a difference between both studies of membranes possibly reflects a difference in their structural characteristics.

The finding that the La^{3+}-induced increase in $2T'_{\parallel}$ or in S is greatest with 5-NS (Table 2) suggests that the fluidity in the vicinity of membrane surface is reduced by binding (or adsorption) of this ion with the membrane surface. Such an inference is concordant with the results shown in Fig. 5, in which a plot of $[La^{3+}]/\Delta 2T'_{\parallel}$ *vs.* $[La^{3+}]$ is shown to be linear and so parallel to the Langmuir plot for La^{3+}-induced inhibition of synaptosomal Ca^{2+}-binding (Kamino *et al.*, 1974). Indeed, nonspecific binding (or deposition) of La^{3+} with cardiac muscle membrane was electronmicroscopically demonstrated (Langer & Frank, 1972). It is expected that a trivalent ion, Ce^{3+}, shows effects quite similar to those of La^{3+}. Heavy metal ions such as Cd^{2+} and Hg^{2+} appear to decrease the fluidity in the vicinity of the surface of membrane phase because of similarity of their effects to those of La^{3+}. Ehrström, Eriksson, Israelachivili and Ehrenberg (1973) also reported that an increase in $2T'_{\parallel}$ of *Bacillus subtilis* membrane labeled with 5-NS was observed in the presence of La^{3+}.

To summarize, the results obtained provide evidence of La^{3+}- and Cd^{2+}-induced change in the fluidity of the surface region of synaptosomal membrane, which appears also consistent with the effect of a heavy metal, osmium tetroxide, on fluidity of nerve membranes of the lobster studied with 5-NS (Jost, Brooks & Griffith, 1973).

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