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A SPIN LABEL STUDY OF RAT BRAIN MEMBRANES
EFFECTS OF TEMPERATURE AND DIVALENT CATIONS

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SUMMARY

Rat brain myelin, synaptosomal plasma membranes and synaptic vesicles were spin labelled with stearic acid nitroxide derivatives. Their electron spin resonance spectra were studied as a function of temperature and divalent ions (Ca^{2+} and Mg^{2+}) concentrations.

(1) Synaptosomal plasma membranes and synaptic vesicles show identical temperature variations of their order parameter ($S = 0.58$ at 35 °C and $S = 0.72$ at 22 °C). Myelin appears more rigid ($S = 0.66$ at 35 °C and $S = 0.76$ at 22 °C). A discontinuity of the order parameter variation as a function of temperature, is observed between 14.5 °C and 19.5 °C with the three types of membranes.

(2) The hydrophobic core of these membranes is very fluid. No transition temperature is observed. The measured values of the spin label rotation correlation times and rotational activation energies are 2.1 and 2.8 ns at 35 °C and 3.1 and 3.6 kcal/mol respectively for synaptosomal plasma membranes and myelin.

(3) Ca^{2+} enhances the membrane rigidity (12 ± 0.7 % increase of the order parameter at 35 °C in the presence of 10^{-3} M Ca^{2+}) and increases the transition temperature. At a lower extend, similar effects are observed with Mg^{2+}.

INTRODUCTION

Numerous studies have been performed which concern the physical state of the lipid components in artificial and natural membranes in relation to their organization and function. These works have been recently reviewed [1, 2, 3]. Numerous variations of the lipidic structure mesomorphic states have been observed as a function of the phospholipid composition and cholesterol contents, or under the influence of physico chemical modifications such as temperature, nature and concentration of metallic ions, interaction with amphiphilic small molecules, etc.

A wide range of physical techniques have been applied to the study of the membrane organization. Among them, the spin label method gives information about the fluidity, the ordering and the mobilities of the lipid components. Thermotropic phase transitions have been observed by this method on numerous artificial systems

and on natural membranes of various origins. Interesting correlations have been found between these lipidic phase transitions and some functional properties of the membrane, for example the activities of certain membrane enzymes [4–8]. Similarly, it has been observed that divalent cations (principally Ca^{2+}) induce a rigidification of artificial membranes [9] and a reorganization of their lipidic components [10–12], particularly when acidic phospholipids are present [13]. It is well known that Ca^{2+} and Mg^{2+} play a fundamental role in many cellular functions, particularly on excitable membranes [14], so it would be interesting to observe if such modifications are also observed with natural nerve membranes.

In the present study, we report results obtained on isolated rat brain membranes (myelin, synaptosomal plasma membranes and synaptic vesicles). Using fatty acid labels, we have observed thermotropic phase transitions which are modified by divalent cations (Ca^{2+} and Mg^{2+}).

MATERIAL AND METHODS

Isolation of brain membranes

Rat brain membranes isolation was performed by the method of Morgan et al. [15, 16]. 200–250 g Wistar rats were decapitated, the brains were immediately homogenized in ice-cold 0.32 M sucrose, and a crude synaptic membrane separation was obtained by low speed differential centrifugation (Sorvall RC 2 B) followed by a first separation on a three-layer ficoll gradient (Spinco L2 50 B, SW 27 rotor), which gave myelin, synaptosomes and mitochondria. An osmotic shock was performed on this crude synaptosome fraction in low ionic strength phosphate buffer (1 mM, pH 7.5/ 10^{-4} M EDTA), and the mixture overlayed on a six-layer sucrose gradient. Six fractions were recovered from the top to the bottom of the tubes: synaptic vesicles, "microsomes", two layers of synaptosomal membranes (fractions F and G), crude synaptosomes, and synaptic mitochondria. Fractions F and G were further purified by high speed differential centrifugation, in 1 mM, pH 7.5 phosphate buffer without EDTA. Two fractions were finally obtained, called "light", and "heavy" synaptic membranes.

The light membrane fraction was a highly purified material, with low myelin and glial contamination as indicated by the analysis of their enzymatic activities [16]. The quality of our preparation was checked by the determinations of acetylcholinesterase [17] and lactic dehydrogenase activities (by following the NADH absorbance at 340 nm in the presence of pyruvate), and by electron microscopy. Protein determinations were made by the method of Lowry et al. [18].

Spin labelling of membranes

Nitroxide fatty acids were purchased from SYVA Corp. Palo Alto and were used without further purification. Spin label I was 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl and spin label II was 2-(10-carboxydecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl. Formulae of these compounds are given on Fig. 1. Stock solutions of the labels were made in dimethylsulfoxide. Membranes (0.9 to 1 mg/ml protein) were labelled by adding 1 % (vol/vol) of these stock solutions in order to obtain a final concentration of 4.10^{-5} M for label I and of 2.10^{-5} M for label II. This last one has a relatively low partition coefficient between membrane and water

[19]. In order to eliminate free label in solution, we have submitted the membranes to a further centrifugation (120 000 × g for 1 h). After decantation, the pellet was resuspended in the same phosphate buffer (1 mM, pH 7.5 without EDTA).

Recording of the ESR spectra

Spin labelled membranes were introduced into a Varian quartz flat cell (sample volume 50 μl), and the spectra were recorded with an E3 Varian Electron spin resonance (ESR) spectrometer. Temperature regulation was obtained with N_2 gas flowing in a Varian cell holder inserted inside the spectrometer cavity. The N_2 gas was cooled by bubbling in a liquid nitrogen tank, then warmed to the working temperature by a resistor. An iron constantan thermocouple inserted in the cell holder gives temperature information to an electronic regulator "thermoelectric model 400". The temperature inside the cell was measured with a chromel alumel fine wire thermocouple (diameter 0.25 mm) connected to an electronic millivoltmeter. Precision on temperature was ± 0.1 °C.

Spectra recorded with label I were interpreted by measuring the order parameter S [20, 21]. An estimation of the rotational correlation time τ_c of spin label II was calculated according to the equation of Henry and Keith [22].

Addition of mono and divalent cations

Stock solutions of $CaCl_2$, $MgCl_2$, KCl, and NaCl were prepared in water. They were added to the membrane samples under a small volume (2 % vol/vol) with a Hamilton syringe, in order to obtain final concentrations ranging from 10^{-6} to 10^{-3} M.

Isolated membranes were stored at +4 °C and studied within 2 days following their preparation. All chemical reagents were commercial products of highest available grade.

RESULTS

A. Effect of temperature

Fig. 1 and 2 show the temperature variations of the ESR spectra obtained with "light" synaptosomal membranes, labelled respectively with I and II. Qualitatively similar modifications were obtained with myelin and synaptic vesicles.

Variation of the order parameter (S) of spin label I. Fig. 3 shows the plot of $\ln S$ as a function of $1/T$ for the three types of membrane studied. Each point is the mean value of the measurements obtained with four different membrane preparations. The spin label is more "immobilized" in myelin than in synaptosomal membranes and in synaptic vesicles which show together the same temperature variation. In each case we observed two straight lines of different slopes, showing a transition temperature between 14.5 and 19.5 °C.

The "heavy" synaptosomal membrane fraction has not been systematically studied. However we have observed that the order parameter at 35 °C ($S = 0.630 \pm 0.030$) is significantly higher for this fraction than for the "light" membranes ($S = 0.570 \pm 0.025$). It is well known that the pH of a buffered solution varies as a function of temperature. In the case of phosphate buffer, this variation is slight in the range of temperature explored here [$(\Delta \mathrm{pH}/\Delta T)/ \simeq -5 \cdot 10^{-3}$ pH/°K]. We have verified that such a pH variation does not affect significantly the order parameter.

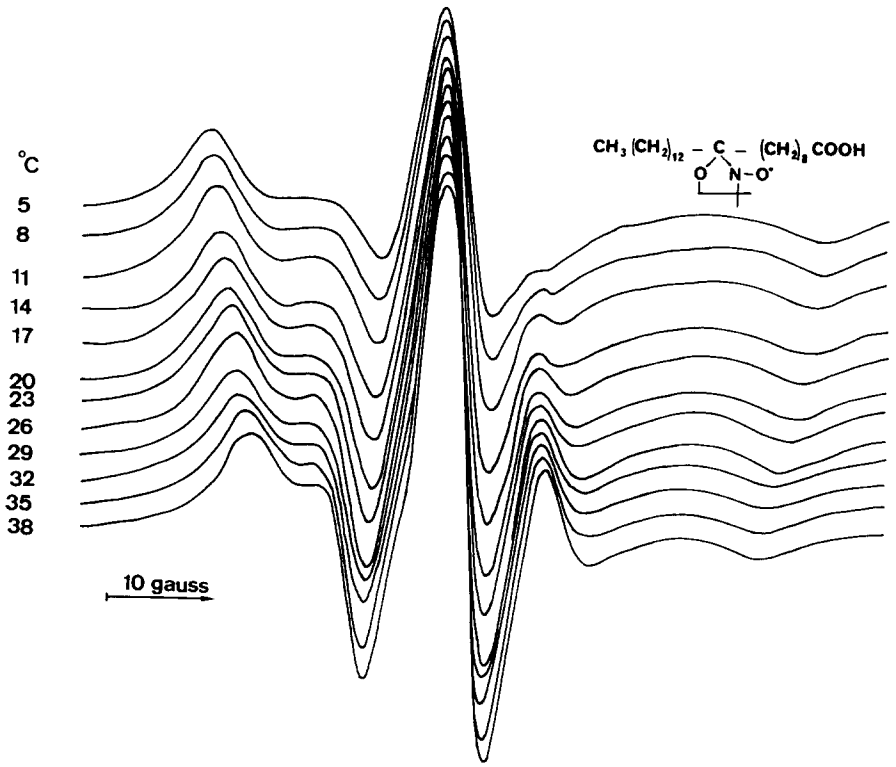

Fig. 1. Temperature dependence of the first derivative ESR spectrum of spin label I incorporated in rat brain synaptic plasma membranes.

Variation of the order parameter (S) *and of the rotational correlation time* (τ_c) *of spin label II.* The spectra obtained with spin label II do not permit us to measure the order parameter in the whole range of temperature studied. At temperatures higher than 20 °C the two high field lines collapse and 2 $T//$ is undetermined.

Fig. 4 shows the plot of ln S as a function of $1/T$ for myelin and for synaptosomal membranes. Both types of membranes show the same temperature variation of their order parameter ($S = 0.270$ at 20 °C and 0.335 at 7 °C).

The spectra obtained with spin label II can be interpreted by the evaluation of the apparent rotational correlation time τ_c according to the formula of Henry and Keith [22].

$$\tau_c = 6.5 \cdot 10^{-10} W_0 \left(\sqrt{\frac{h_0}{h_{-1}}} - 1 \right)$$

were W_0 is the linewidth of the central line and h_0 and h_{-1} are respectively the amplitudes of the central and of the high field lines. The variation of ln τ_c as a function of $1/T$ is shown on Fig. 4, in the case of myelin and synaptosomal membranes. Both plots give straight lines in the whole range of temperature studied. The correlation time is higher in myelin (2.8 ns at 22 °C) than for synaptosomal membranes (2.1 ns).

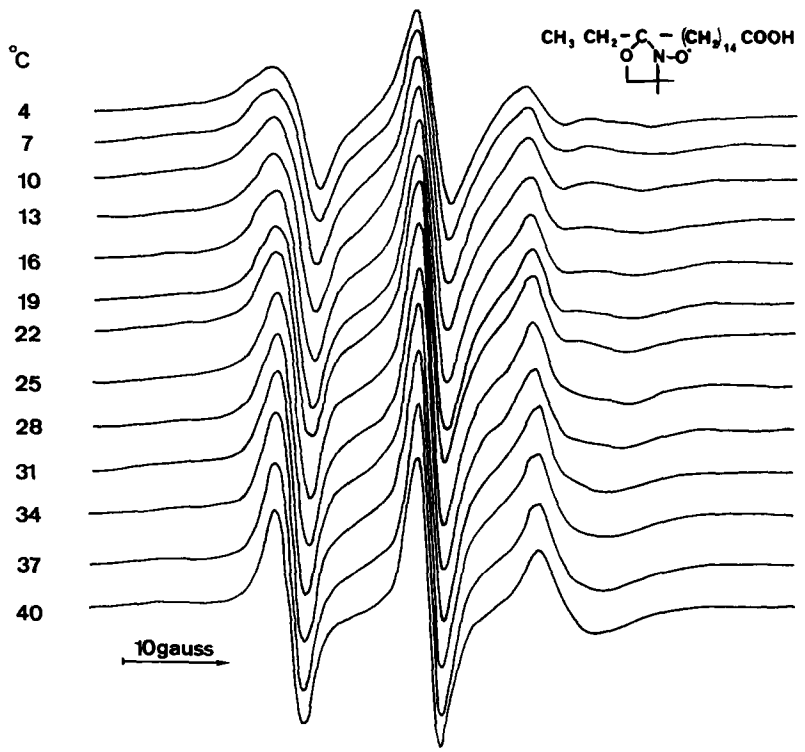

Fig. 2. As in Fig. 1 for spin label II.

Calculations of the rotational activation energies of spin label II included in the lipidic phase give the following results: 3.6 kcal/mol for myelin and 3.1 kcal/mol for synaptosomal membranes. It was not possible to obtain exploitable data with synaptic vesicles for this material was obtained with a too low yield.

B. Effect of metallic ions

Effect of temperature in the presence of Ca^{2+}. In the presence of 10^{-3} M Ca^{2+}, the order parameter of spin label I in synaptosomal membranes is increased at all temperatures studied (Fig. 5). The transition temperature is now observed at 22.5 °C. With spin label II no modification of the correlation time and of the order parameter were observed in the whole range of temperature studied (Fig. 4).

Effect of Ca^{2+} *concentration.* We have studied the order parameter variation of spin label I as a function of the Ca^{2+} concentration at 35 °C, on the three types of isolated membranes. Results are presented on Fig. 6. Same results are observed with synaptosomal membranes (light fraction) and synaptic vesicles: the S value increases significantly for $5 \cdot 10^{-5}$ M Ca^{2+}; at 10^{-3} M, this increase reaches 12 ± 0.7 % (Fig. 7). With myelin or with the "heavy" synaptosomal membrane fraction, the effect of Ca^{2+} is less important (4% increase at 10^{-3} M Ca^{2+} for myelin and 7% with "heavy" membranes).

Effect of Mg^{2+}, K^{+} *and* Na^{+}. The effect of these ions was studied on "light"

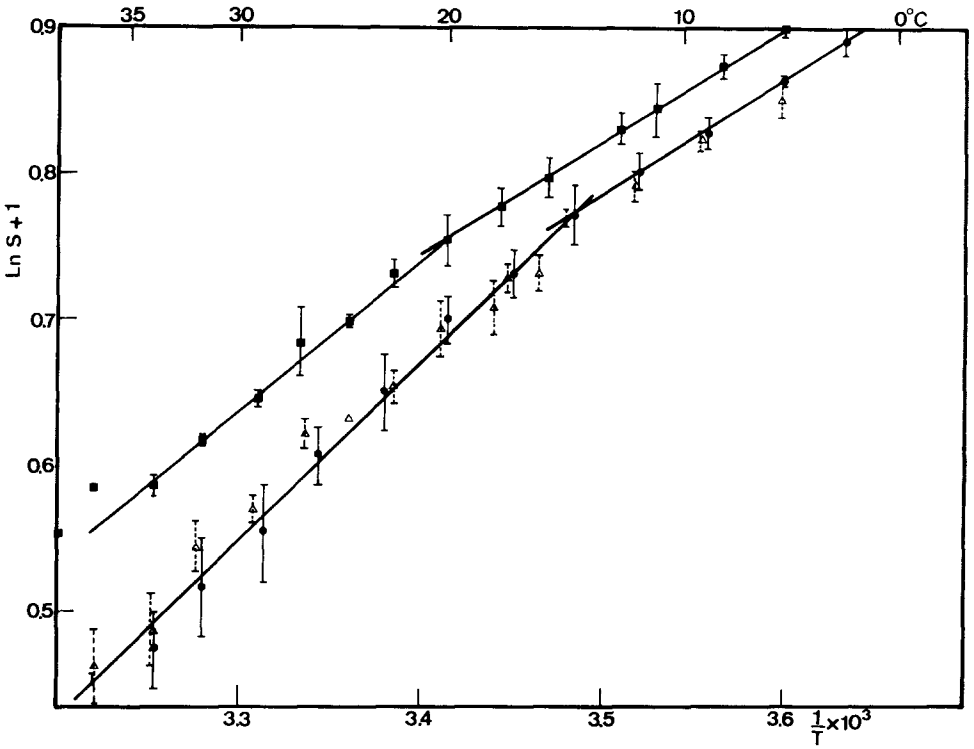

Fig. 3. Temperature variation of the order parameter S (spin label I) ■, myelin; ●, synaptic plasma membranes; △, synaptic vesicles.

synaptosomal membranes at 35 °C. It can be shown in Fig. 7 that Mg^{2+} is slightly less effective than Ca^{2+} (9.5% increase of S with 10^{-3} M Mg^{2+}). K^+ and Na^+ behave similarly: a moderate rigidification is observed only at high concentrations (5% increase of S at 10^{-2} M).

DISCUSSION

Effect of temperature on the spin label motion

Spin label I is sensitive to changes in the polar part of the phospholipid structure [23]. The order parameter S or the experimental hyperfine splitting $2T_{//}$ have been measured on numerous biological systems. Table I summarizes the principal results found in the literature. Although significant variations are observed between these different membranes, it can be seen that their polar part is rigid and highly organized. The order parameter measured with myelin is among the highest, and the mobility of spin label I is identical in synaptic membranes and vesicles. Both are less rigid than myelin. The significant difference observed between "light" and "heavy" synaptosomal membrane fraction can be due either to a difference in their lipidic composition, or to a residual contamination by myelin [16].

Spin label II explores the hydrophobic part of the lipidic membrane structure.

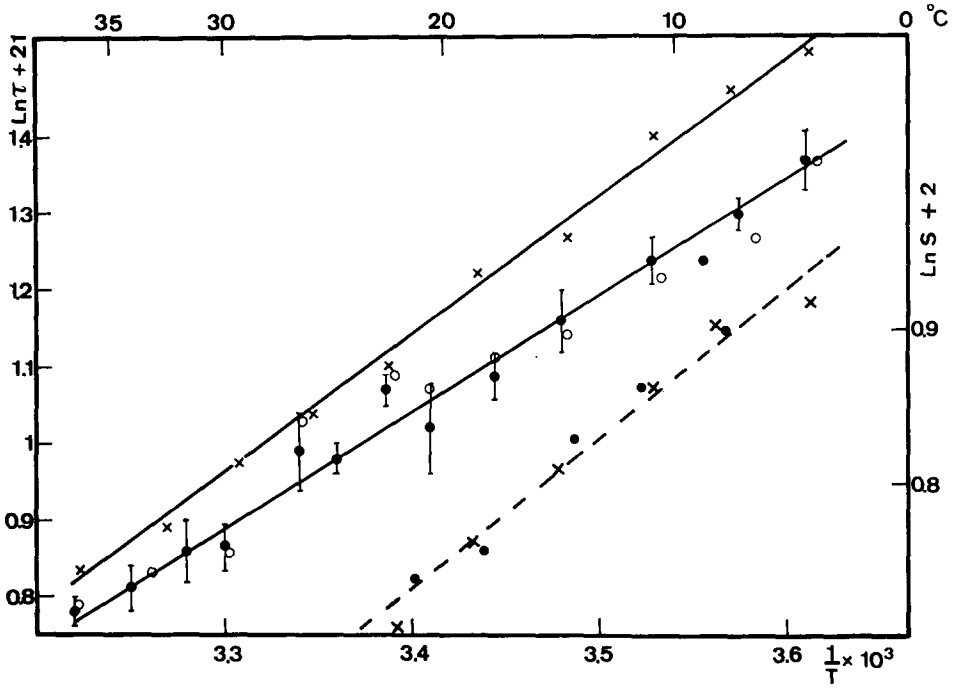

Fig. 4. Temperature variation of the rotational correlation time and the order parameter S of spin label II: ×, myelin; ●, synaptic membranes without Ca^{2+}; ○, synaptic membranes in the presence of 10^{-3} M Ca^{2+}. Dashed line is related to the order parameter.

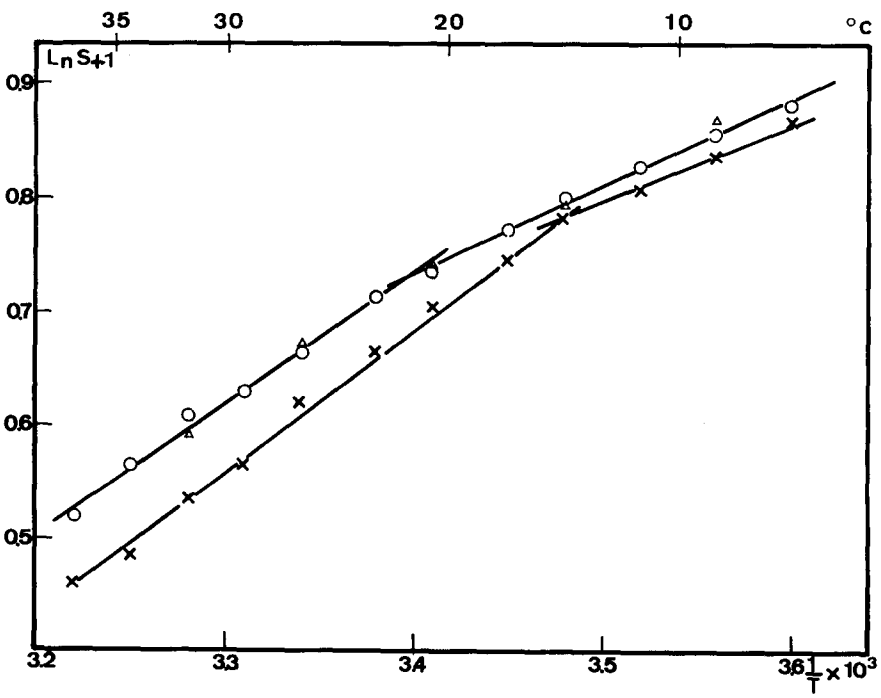

Fig. 5. Temperature variation of the order parameter of synaptic membranes: ×, without Ca^{2+}; ○, in the presence of 10^{-3} M Ca^{2+}, temperature is increasing; △, in the presence of 10^{-3} M Ca^{2+}, temperature is decreasing.

TABLE I

PRINCIPAL RESULTS CONCERNING THE EFFECTS OF TEMPERATURE ON BIOLOGICAL MEMBRANES, USING THE SPIN LABEL METHOD

Spin label I is generally *N*-oxyloxazolidine derivative of stearic or ketostearic acid substituted in position 3 relative to the carboxyl. It labels the polar part of the lipidic structure. Spin label II is generally the stearic acid substituted in position 14 relative to the carboxyl. It labels the hydrophobic core of the lipidic structure. θ is the transition temperature. τ_c are given in nanosecond. E_A (kcal/mol) are given in the order of decreasing temperatures.

Ref.	Material	Spin label I							Spin label II						
		S (35 °C)	$2T_{\parallel}$	S (22 °C)	$2T_{\parallel}$	S (5 °C)	$2T_{\parallel}$	θ (°C)	35 °C	22 °C (τ_c ns)	5 °C	θ (°C)	E_A (kcal/mol)		
4	Rat liver mitochondria								2.4	2.8	6.3	24-8	2.7	5.8	6.4
8	Rat liver mitochondria								4.5	4.7	8	23	3.1	4.8	
4	Sheep liver mitochondria								0.6	1.0	2.2	29-17	3.7	4.1	6.4
8	Fish liver mitochondria								1.0	1.5	2.8	none		5.6	
8	Potato mitochondria								2.8	4.2	8.5	none		6.8	
8	Sweet potato mitochondria								4.0	4.5	9.0	12	3.1	4.8	
24	Brown adipose tissue mitochondria	0.58		0.68		0.80		none							
25	Squirrel liver mitochondria														
	active								0.3	0.7	1.0	20			
	hibernat								1.0			none			
4	Rat liver microsomes								2.2	2.7	7.1	22- 7	5.1	7.2	9.8
4	Sheep liver microsomes								0.9	1.3	2.5	33-11	4.4	5.2	5.6
6	Guinea lig liver microsomes			0.61		0.74									

26	Erythrocyte ghosts		52.2		56		60.3	30						
27	*Mycoplasma laidlawii* [1]		52		58		64	none						
27	*Mycoplasma laidlawii* [2]		50		59		64	≃ 10						
27	*Mycoplasma laidlawii* [3]		49		54		63	(?)						
28	*Mycoplasma laidlawii* [4]									4.0	6.0	40	3.9	5
28	*Mycoplasma laidlawii* [5]									2.6	4.1	none	4.1	
29	*Acholeplasma laidlawii*		56		60		63.5	none						
29	*Mycoplasma hominis*		52		56.5		63	≃ 7						
30	*E. Coli* vesicles	0.59		0.61		0.81 (10 °C)		20–30	1.2	1.5	2.2 (10°)	20-31	4.4	2.2
31	*E. coli* membranes [6]	0.62		0.82				29						
31	*E. coli* membranes [7]	0.62		0.78				23.5						
	E. coli membranes [8]							14						
32	*Saccharomyces cerevisiae* (KD 46) [9]								1.0	1.8	3.2	22		
32	*Saccharomyces cerevisiae* (KD 46)[10]								0.8	1.4	2.8	12		
32	*Saccharomyces cerevisiae* (KD 46) [11]								0.7	1.1	2.5	16		
	Myelin	0.66	53.5	0.76	57	0.90	60.7	19.5	1.8	2.8	3.4	none	3.6	
	Synaptic vesicles	0.58	48.2	0.72	55.5	0.86	60	14.5						
	Synaptic plasma membranes [12]	0.58	48.2	0.72	55.5	0.86	60	14.5	1.7	2.1	2.9	none	3.1	
	Synaptic plasma membranes [13]	0.62		0.75		0.88		22	1.7	2.1	2.9	none	3.1	

[1] Natural *M. laidlawii* membranes. [2] *M. laidlawii* enriched with *trans*-Δ^9 octadecenoïc acid. [3] *M. laidlawii* enriched with *cis*-Δ^9 octadecenoïc acid. [4] Stearate enriched membranes. [5] Oleate enriched membranes. [6] *Trans*-18:1 enriched *E. coli*. [7] *Trans*-16:1 enriched. [8] *Cis*-18:1 enriched. [9] 76% *cis*-Δ^3 octadecanoate enriched *S. cerevisiae*. [10] 78% *cis*-Δ^9 octadecanoate enriched. [11] 62% *cis*-Δ^6 octadecanoate enriched. [12] Synaptic plasma membrane without Ca^{2+}. [13] Synaptic plasma membrane in the presence of 1 mM Ca^{2+}.

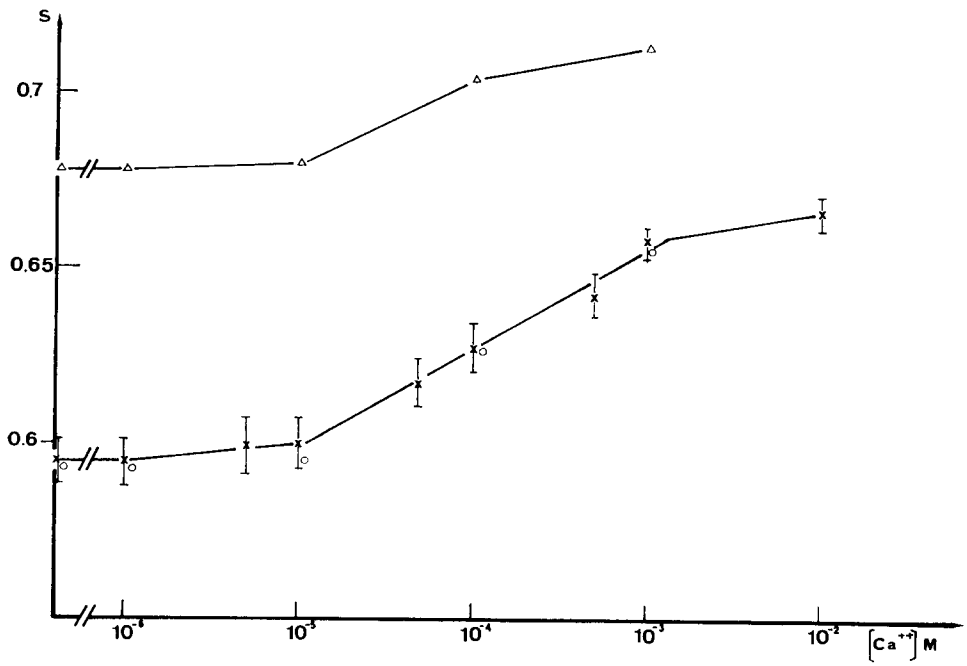

Fig. 6. Variation of the order parameter S as a function of the Ca^{2+} concentration. ×, synaptic membranes; ○, synaptic vesicles; △, myelin. The S values at zero Ca^{2+} concentration were measured in presence of 10^{-3} M EDTA.

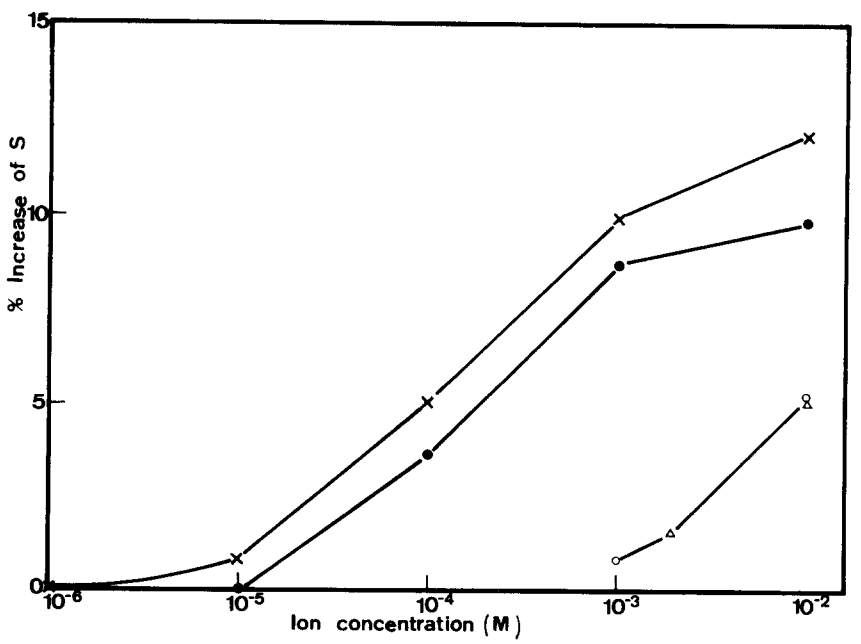

Fig. 7. Relative increase of the order parameter S of synaptic membranes (spin label I) as a function of the concentration of ions. ×, Ca^{2+}; ●, Mg^{2+}; ○, K^{+}; △ Na^{+}.

The order parameter S is generally not measurable on the spectra obtained with this spin label, at least at temperatures higher than approximatively 20 °C. These spectra are thus generally interpreted by calculating the rotational correlation time of the spin label using the formula of Henry and Keith [22]. It must be emphasized that this formula is valid only in case of a spheric nitroxide molecule moving in an isotropic medium and for $\tau_c < 10^{-9}$ s. These conditions are not satisfied in the case of 16 doxyl stearic acid in membranes. However this simple equation for τ_c offers a mean of evaluation of the spin label mobility. Furthermore, in spite of its lack of solid theoretical justification, this formula has been used by numerous authors on a great variety of membranes, and comparisons between these membranes are thus possible.

As seen in Table I, wide variations in correlation time values have been reported. In our experimental conditions, the hydrophobic cores of rat brain membranes show a fluidity which is comparable to that of rat liver mitochondria or microsomes. It seems interesting to note that the correlation times measured with myelin and with synaptic membranes are not very different. Furthermore the order parameter values (between 4 and 20 °C) are the same for both types of membranes. Myelin is thus characterized by a high fluidity gradient, between its polar and hydrophobic parts.

Table I summarizes also the temperature dependent responses observed with spin labels on various biological membranes. These studies have been performed more often with label II than with label I and only in a few cases with both labels on the same material. Transition temperatures are observed in a range from 7 to 40 °C and vary as a function of the nature of the material and of their lipidic composition. They are most often observed as a sudden change in the temperature dependent variation of the order parameter S or of the correlation time τ_c. Such transitions are observed in the present study between 14.5 ° and 19.5° C with the three types of membranes. Statistical analysis of the experimental lines (linearity test) does not permit us to be sure that the discontinuity observed at 19.5 °C for myelin is significantly higher than the observed value of 14.5 °C for synaptic membranes and vesicles. The observation of a "transition temperature" in the case of myelin is somewhat surprizing since this membrane is known to show no thermal transition when studied by differential scanning calorimetry [1, 33]. However the changes in membrane thermal behavior observed with spin labels are not always superimposable with those studied by other methods [8]. This can be due either to a modification of the membrane structure caused by the incorporation of the spin labels (particularly in the hydrophilic part) [34] or to a localized mesomorphic change not detectable by calorimetric studies.

As the lipidic composition of these natural membranes is complex, their phase behaviour must be characterized by at least two break points corresponding to complete melting and complete solidification. Our results show only one discontinuity. Two explanations can be given for this fact. First the complete melting is perhaps observable at temperatures higher than 40 °C but the membrane structure can be altered at such high temperatures. Secondly, it is possible that above the lower melting point the nitroxide fatty acids partition prefentially into the fluid phase, making the upper boundary inobservable.

The high value of the order parameter in the polar part of myelin lipids and the relatively low values observed in synaptic membranes and vesicles can be related to their different lipidic composition. A high proportion of polyenoïc fatty acids are

found in synaptic membranes and vesicles [35, 36], whereas they do not contain the long chain fatty acids characteristic of myelin. In many cases, [27, 28, 31, 32], it has been shown that enrichment of natural membranes with unsaturated fatty acids decreases the order parameter. On the other hand, the identical temperature dependance of the spin label response observed with synaptic membranes and vesicles is well explained by their similarity in lipidic composition [36].

This identity in the lipidic physical chemical state of synaptic vesicles and membranes can be used as a favorable argument for the hypothesis of the exocytosis-endocytosis cycle of synaptic vesicles inside the central nervous system [37].

No variation of the slope in the plot of τ_c against $1/T$, was observed with spin label II. The low measured values of the apparent correlation time, of the order parameter and of the energy of activation confirm previously obtained results that the hydrophobic core of these nervous membrane is very fluid [21, 23, 38].

Effect of divalents ions

The enhancement of the membrane rigidity observed with Ca^{2+} and Mg^{2+} is not due to the increase in the ionic strength of the solution, since Na^+ and K^+ are without effect at 10^{-3} M (ionic strength 10^{-3}) whereas divalent ions are effective at 10^{-4} M (ionic strength 3.10^{-4}).

Analogous effects have been observed [39] on *Escherichia coli* vesicles at relatively high ionic concentrations.

Madeira and Antunes-Madeira [40] have shown an increase in the fluorescence response of 8-anilinonaphthalene 1-sulphonate labelled nerve membranes under the influence of Ca^{2+}. Our results with spin labels show that synaptic membranes and vesicles are very sensitive to Ca^{2+} and Mg^{2+} since a significant increase of the order parameter is observed with a concentration as low as 5.10^{-5} M. This effect is localized in the polar part of the lipidic structure since no modification is observable with spin label II. Furthermore, the rigidity increase seems to induce a modification in the thermal transition of the membrane. The change in spin label temperature response variation increases from 14.5 °C in absence of Ca^{2+} to 22 °C in presence of 10^{-3} M of this ion. Numerous works have shown that divalent cations, particularly Ca^{2+}, rigidify artificial lipidic structures [1, 9, 13, 41] and modify the spatial distribution of their phospholipids [10, 12, 42, 43]. These conformational changes of the membranes are observed when acid phospholipids are present.

Synaptic plasma membranes and vesicles are known for their high content in phosphatidyl ethanolamine (35 %) phosphatidylserine (15 %) and phosphatidyl inositol (4 %) [35, 36]. By reference to the modifications induced in model systems it is probable that Ca^{2+} induces similar changes in the structure of brain membranes. However, synaptic membranes and vesicles are approximately 10-times more sensitive to the effect of divalent cations than artificial membranes. This difference can be due to the presence of proteins, which are known to shift down the temperature of the gel-liquid crystal transition of the lipids [44].

The slight rigidification observed on myelin with Ca^{2+} could be due to the presence of synaptic membranes in our preparation, which was not performed in order to obtain ultra pure myelin.

The important structural modification induced by divalent cations at the lipidic level of the brain membranes can have interesting implications in the understanding

of the role of these ions. Rigidification of the membrane modifies its ionic permeability [45], it can induce changes in the relative organization and reactivity of membrane bound proteins, particularly of enzymes and receptors [42, 46]. Modifications of the membrane structures have been observed under the effect of local anaesthetics, which are able to displace membrane bound Ca^{2+}, [47, 48], similar effects could explain the membrane action of other drugs, particularly tranquillizers [49–52].

ACKNOWLEDGEMENTS

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