Orientation and Motion of Spin-Labels in Rabbit Small Intestinal Brush Border Vesicle Membranes†

Helmut Hauser,* Nigel Gains, Giorgio Semenza, and Martin Spiess

ABSTRACT: The temperature dependence of the packing (order) and fluidity (microviscosity) of rabbit small intestinal brush border vesicle membranes and of liposomes made from their extracted lipids has been investigated by using a variety of lipid spin probes. The lipids in the brush border membrane are present essentially as a bilayer. Compared to other mammalian membranes, the brush border membrane appears to be characterized by a relatively high packing order as well as microviscosity. At body temperature, the lipid molecules undergo rapid, anisotropic motion, which is essentially a fast rotation about an axis approximately perpendicular to the bilayer normal. Both the order (motional anisotropy) and the microviscosity increase with decreasing temperature and with increasing distance from the center of the bilayer. Qualitatively similar motional or fluidity gradients have been reported for other mammalian and bacterial membranes. The liposomes made from the extracted lipids have a somewhat lower packing order and a slightly higher fluidity than brush border vesicle membranes. The differences are, however, small, indicating that the packing and the fluidity (microviscosity) of the membrane are primarily determined by the lipid composition. Membrane-associated proteins and cytoskeleton cannot play a dominant role in determining the order and fluidity of the lipid bilayer. Discontinuities are observed in the temperature dependence of various spectral parameters, the order parameter S, the rotational correlation time τ, and 2,2,6,6-tetramethylpiperidinyloxy partitioning. They are assigned to phase transitions and/or phase separations of the membrane lipids. These discontinuities occur at about 30, 20, and 13 °C for 5-doxyl-, 12-doxyl-, and 16-doxylstearic acid, respectively. The apparent transition temperature depends on the location of the spin probe along the bilayer normal, being higher the closer the probe is to the membrane surface. This indicates the possibility that chain melting is progressive and spreads with increasing temperature from the center of the membrane outward.

The microvillus plasma membrane of the small intestine is highly specialized for digestion and absorption. The fluidity and temperature-dependent phase transitions of the lipid part of the bilayer have been shown to influence the activity of some integral membrane enzymes and transport proteins. Membrane fluidity and the temperature-dependent phase transitions of brush border vesicles, from rat intestine, have previously been investigated by using both fluorescence polarization and differential scanning calorimetry (Schacter & Shinitzky, 1977; Brasitus et al., 1979; Brasitus & Schacter, 1980). Both the native membranes and liposomes prepared from their extracted lipids were studied.

The work described in this paper extends these studies to brush border vesicles from rabbit small intestine and to liposomes made from lipids extracted from these membranes. Membrane and lipid bilayer fluidity (microviscosity) was probed by ESR spin-labeling.

Materials and Methods

The following spin-labels were purchased from Syva (Palo Alto, CA): 2,2,6,6-tetramethylpiperidinyloxy (Tempo);[1] the cholesterol analogue 4′,4′-dimethylspiro(5α-cholestan-3,5′-oxazolidin)-3′-yloxy (cholestane spin-label); stearic acids with the 4,4-dimethyl-3-oxazolidinyloxy group (doxyl group) attached to either carbon atom 5 (5-doxylstearic acid, 12 (12-doxylstearic acid), or 16 (16-doxylstearic acid). Spin-labeled phosphatidylcholine, 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-phosphatidylcholine (5-doxyl-PC), was prepared by starting from dipalmitoyl-*sn*-phosphatidylcholine, which was synthesized according to Paltauf et al. (1971). Lysophosphatidylcholine was then obtained by hydrolysis with phospholipase A_2 by standard methods (Chakrabarti & Khorana, 1975) and the lyso compound was acylated with 5-doxylstearic acid anhydride, according to Gupta et al. (1977).

Brush border membrane vesicles were prepared from rabbit small intestine, which had been stored at −50 °C (Schmitz et al., 1973; Kessler et al., 1978; Gains & Hauser, 1981). However, the procedure was modified according to Hauser et al. (1980) in that the isolation medium consisted of 300 mM D-mannitol, 5 mM EGTA, and 5 mM Hepes plus KOH to pH 7.6, and further that 10 mM $MgCl_2$ was used in the precipitation step. The inclusion of EGTA and the replacement of $CaCl_2$ by $MgCl_2$ in the precipitation step are essential if on extraction of brush border vesicle lipids calcium-stimulated lipolysis is to be minimized (Hauser et al., 1980).

Lipids were extracted from 1 volume of brush border vesicles (21 mg/mL) with 19 volumes of chloroform/methanol (2:1 v/v); after filtration, 5 volumes of this extract was washed twice with 1 volume of 0.37% KCl as described by Folch et al. (1957). After rotary evaporation of the organic phase, the lipids were dissolved in chloroform. A total of 100 mg of lipid was extracted from brush border vesicles having a protein content of 180 mg as measured with the Bio-Rad protein assay (Bio-Rad Laboratories, 8152 Glattburg, Switzerland) by using bovine serum albumin as the standard (Bradford, 1976).

The fatty acid and cholesterol spin-labels were incorporated into brush border vesicles by using the following procedure. The spin-labels were distributed over the surface of a round-

† From the Laboratorium für Biochemie, Eidenössische Technische Hochschule Zürich, ETH-Zentrum, CH 8092 Zürich, Switzerland. *Received March 29, 1982.* This work was supported by the Swiss National Science Foundation (Grant 3.570-0.79).

[1] Abbreviations: PC, phosphatidylcholine; Tempo, 2,2,6,6-tetramethylpiperidinyloxy; EDTA, ethylenediaminetetraacetic acid, disodium salt; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/82/0421-5621$01.25/0

bottomed flask by rotatory evaporation at room temperature (21 ± 2 °C); when dry, the temperature was raised to 50 °C for 5 min. After the labels were cooled to room temperature, the brush border vesicle suspension was added and rotated for 10 min for fatty acid incorporation and for 30 min for cholesterol incorporation. The latter was incorporated to an extent of about 75%, the unincorporated label remaining on the glass wall. Spin-labeled liposomes were prepared by evaporation of $CHCl_3$/MeOH (2:1 v/v) from a solution containing the spin-label and the lipid extract followed by dispersion of these lipids in the isolation medium (300 mM D-mannitol, 5 mM EGTA, and 5 mM Hepes plus KOH to pH 7.6) with a glass bead.

Spin-labeled PC was incorporated into brush border vesicle membranes by using PC exchange protein by the procedure of Barsukov et al. (1980). The PC exchange protein was prepared from beef liver according to Kamp et al. (1973) except that the last purification step was omitted. Dispersions of egg PC mixed with spin-labeled PC (molar ratio 4:1, PC concentration 0.25 mM in 300 mM D-mannitol, 1 mM EDTA, and 10 mM Hepes plus KOH to pH 7.6) were prepared as described by Brunner et al. (1978). The dispersion was sonicated for 10 min (Branson B 30 with a microtip) and centrifuged at 27000*g* for 30 min. The spin-label was not affected by this procedure as judged from the spectral intensity before and after sonication. Brush border vesicles containing 0.8 μmol of PC (0.6 mg of PC) and 8.5 mg of protein in 0.5 mL were added to a mixture containing 0.2 mL of the spin-labeled PC dispersion and 80 μg of PC exchange protein in 0.3 mL; this was then incubated at 37 °C for 30 min. The reaction was stopped by cooling the sample in ice/H_2O. The brush border vesicles were separated from the liposomes by centrifugation at 27000*g* for 30 min. The pellet was washed by redispersing it in buffer followed by centrifugation as described above. As 50–60% of the PC is transferred from the liposomes to the brush border vesicles, then the molar ratio of spin-labeled PC to total lipid in the brush border vesicle membrane is ~1:300.

In a parallel experiment, brush border vesicles were incubated with liposomes doped with [^{3}H]dipalmitoyl-PC and [^{14}C]cholesteryl oleate, as a nonexchangeable marker; otherwise, the conditions were the same as described above. These experiments showed that all the PC accessible on the outer leaflet of the liposomes is exchangeable in the presence of phospholipid exchange protein. Further, when brush border vesicles were incubated with liposomes in the absence of exchange protein, 10–15% of the liposomal lipid (equivalent to 2–3% of the total phosphatidylcholine of the brush border vesicle suspension) remained associated with the brush border vesicles and could not be separated from them by centrifugation (as described above). That the liposomal lipid did not fuse with the brush border membrane could be seen from the ESR spectrum. This spectrum was qualitatively similar to that of the original liposomes (before incubation with brush border membranes) and is characteristic of spin exchange. The contribution of this contamination to the ESR spectrum of the spin-label that had been incorporated into the brush border vesicle membrane in the presence of the exchange protein is negligible.

ESR spectra were recorded at 9.2 GHz with a Varian X-band spectrometer (Model E-104A) fitted with a variable temperature device. Samples, 20–50 μL, were measured in glass capillary tubes of 1-mm internal diameter. The temperature was monitored with either a thin thermometer or a small thermocouple before and after each spectrum was recorded and was accurate to ±0.5 °C. The microwave power was maintained at 20 mW, and the amplitude of the 100-kHz field modulation was less than one-third of the narrowest peak to peak line width. The magnetic field sweep (usually 100 G) was calibrated relative to a dilute solution of Fremy's salt (a_N = 13.09 G). For the spin-labels undergoing rapid anisotropic motion (5- and 12-doxylstearic acid and 5-doxyl-PC), the order parameter S was calculated provided the inner and outer hyperfine peaks were well resolved from each other. The order parameter S was calculated according to Hubbell & McConnell (1971) and Seelig (1976):

$$S = \frac{1}{2}(3\,[\cos^2\theta] - 1) = \left(\frac{T_\parallel - T_\perp}{T_{zz} - T_{xx}}\right)\frac{a_0}{a'} \qquad (1)$$

$[\cos^2\theta]$ is the mean angular deviation of the nitrogen 2p π orbital from the director axis of the bilayer, i.e., from the bilayer normal. $T_\parallel$ and $T_\perp$ are half the separation of the outer and inner hyperfine splittings, respectively. The factor a_0/a' is a correction of the polarity dependence of the hyperfine splittings $a_0 = {}^1/_3(T_{xx} + T_{yy} + T_{zz})$ and $a' = {}^1/_3(T_\parallel + 2T_\perp)$. The values for the principal components T_{xx}, T_{yy}, and T_{zz} of the hyperfine splitting tensor, 5.8, 5.8, and 30.8 G, respectively, were taken from the work of Hubbell & McConnell (1971). For 16-doxylstearic acid, which undergoes rapid and essentially isotropic motion (τ usually $<3 \times 10^{-9}$ s), the orientational correlation time τ was calculated according to the theory of Kivelson (1960) (Stone et al., 1965):

$$\tau = (6.5 \times 10^{-10})W_0[(h_0/h_{-1})^{1/2} - 1] \qquad (2)$$

where W_0 is the peak to peak width of, and h_0 is the height of, the center line ($m_I = 0$) and h_{-1} is the height of the high-field line ($m_I = -1$).

The distribution of Tempo between the membrane and the aqueous phase was monitored as described by Shimshick & McConnell (1973); the Tempo spectral parameter [$f = H/(H + P)$; see Figure 6A] gives to a first approximation the fraction of Tempo dissolved in the membrane (Hubbell & McConnell, 1968; Shimshick & McConnell, 1973). For comparison of the distribution of Tempo into brush border vesicles and into liposomes, the parameter f was divided by the lipid concentration in grams per milliliter (Tomkiewicz & Corker, 1975).

For the ESR experiments, the labeled brush border vesicles and liposomes were kept on ice, and all measurements were carried out during the course of 1 day. For measurements at temperatures above 5 °C, a fresh sample was used for each spectrum recorded.

Results

For the fatty acid spin-labels used in this study (5-, 12-, and 16-doxylstearic acid), it was found that at a given temperature and for a given spin-label the ESR spectra were qualitatively very similar for the spin-label incorporated into brush border vesicle membranes and into liposomes made from their extracted lipids. The spectra in Figure 1A are of the labels incorporated into brush border vesicle membranes; they were recorded between 38 and 41 °C. The spectra in Figure 1B are of the same labels but incorporated into the liposomes and recorded between 3 and 4 °C. The spectra of 5- and 12-doxylstearic acid (Figure 1) are typical for lipid dispersions in which the label is undergoing rapid but anisotropic motion. Values for the hyperfine splitting $2T_\parallel$, which is an indication of the anisotropy of motion, are given in Table I. The $2T_\parallel$ values measured in the liposomes are smaller than those obtained for the brush border vesicle membranes, indicating that the motional anisotropy is reduced in the liposomes. The $2T_\parallel$

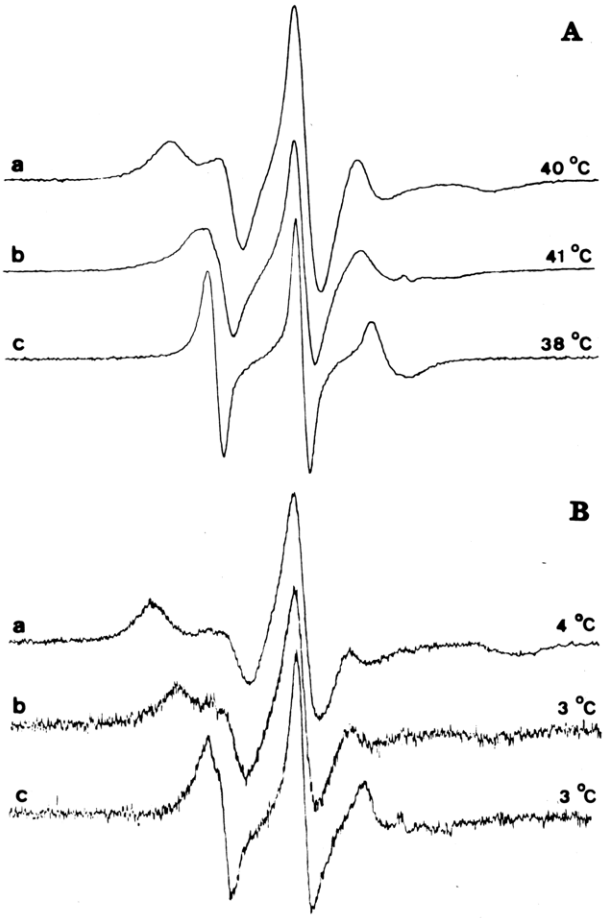

FIGURE 1: (A) ESR spectra of 5-, 12-, and 16-doxylstearic acid (spectra a, b, and c, respectively) incorporated into brush border vesicle membranes dispersed in 0.3 M D-mannitol, 5 mM EGTA, and 5 mM Hepes plus KOH to pH 7.6. The protein concentration was (a) 15 mg/mL (~8.5 mg of lipid/mL, lipid: spin-label molar ratio 200:1) and (b and c) 27 mg/mL (~15 mg of lipid/mL, lipid:spin-label molar ratio 100:1). (B) ESR spectra of 5-, 12-, and 16-doxylstearic acid (spectra a, b, and c, respectively) incorporated into lipids extracted from brush border vesicles and dispersed in the same buffer as in (A). The lipid concentrations were (a) 13.8, (b) 16.3, and (c) 9.2 mg/mL. The lipid:spin-label molar ratios were 100:1.

Table I: Hyperfine Splitting Constants, $2T_{\parallel}$ and $2T_{\perp}$, of Various Spin-Labels in Brush Border Vesicle Membranes and Liposomes Made from Their Extracted Lipids[a]

		vesicles		liposomes	
spin-label	temp (°C)	$2T_{\parallel}$[b] (G)	$2T_{\perp}$ (G)	$2T_{\parallel}$ (G)	$2T_{\perp}$ (G)
5-doxylstearic acid	4	64.3	c	61.0	c
	25	57.3	18.3	54.0	19.0
	40	53.3	19.4	51.0	20.2
5-doxyl-PC	4	64.5	c	64.2	c
	25	58.6	16.8		
	40	51.5	18.5	52.5	19.1
12-doxylstearic acid	4	60.0	c	53.0	c
	25	45.8	19.8	44.2	20.2
	40	41.0	21.3	40.0	21.5

[a] Values were taken from plots of the temperature dependence of $2T_{\parallel}$ and $2T_{\perp}$ (plots not shown). [b] To a first approximation, $2T_{\parallel}$ is used as a measure of the motional anisotropy since $2T_{\perp}$ and hence the order parameter S cannot be determined at low temperatures. [c] Not determinable.

values for 5-doxyl-PC are similar to those observed with 5-doxylstearic acid. The ESR spectra of 5-doxyl-PC in brush border vesicle membranes as a function of temperature are shown in Figure 2. The appearance of the spectra of this label at temperatures below 20 °C approached that of a powder

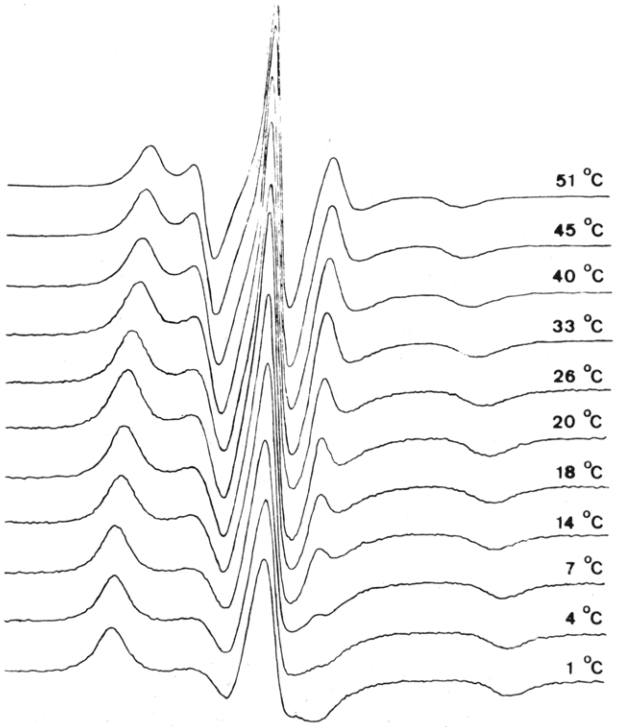

FIGURE 2: Temperature dependence of the ESR spectra of 5-doxyl-PC incorporated into brush border vesicle membranes dispersed in 0.3 M D-mannitol, 1 mM EDTA, and 10 mM Hepes plus KOH to pH 7.6. The vesicle concentration was 20 mg of protein/mL (~11 mg of lipid/mL); the lipid:5-doxyl-PC molar ratio was ~300:1.

Table II: Hyperfine Splitting Constant, a_N,[a] at 25 °C, of Spin-Labels Incorporated into Brush Border Vesicle Membranes and Liposomes Made from Their Extracted Lipids Compared with the Values Found for Other Membranes

	a_N (G)		
	5-doxylstearic acid	12-doxylstearic acid	16-doxylstearic acid
brush border vesicles[b]	15.3 ± 0.1	14.2 ± 0.1	13.5 ± 0.05
liposomes from above[b]	15.3 ± 0.2	14.1 ± 0.1	13.5 ± 0.05
beef heart mitochondria[c]	15.4 ± 0.1		
egg PC in H_2O	15.1 ± 0.1	14.3 ± 0.1	14.1 ± 0.2
microsomes[d]	15.1	14.6	14.4
liposomes from above[d]	15.1	14.5	14.4

[a] In the case of anisotropic motion, a_N was calculated according to $a_N = (T_{\parallel} + 2T_{\perp})/3$ (Seelig, 1976). [b] Experimental conditions given in the legend to Figure 1. [c] In 10 mM phosphate buffer, pH 7.4. [d] Data from Griffith et al. (1974).

spectrum with $2T_{\parallel} \rightarrow 2T_{zz}$ (Figure 2). A similar behavior was observed for 5-doxylstearic acid.

In Table II, values for the hyperfine splitting constant a_N of various lipid spin-labels incorporated into brush border vesicle membranes and the liposomes are given. The hyperfine splitting constant a_N is a measure of the polarity of the spin-label environment, and within experimental error, the same a_N values were measured for the labels incorporated into the brush border vesicles as into the liposomes. These values are in good agreement with data in the literature (Seelig & Hasselbach, 1971; Esser & Lanyi, 1973; Griffith et al., 1974), with the exception of the value for 16-doxylstearic acid, which appears to be lower. 5-Doxyl-PC appears to be in the same environment as the corresponding 5-doxylstearic acid label.

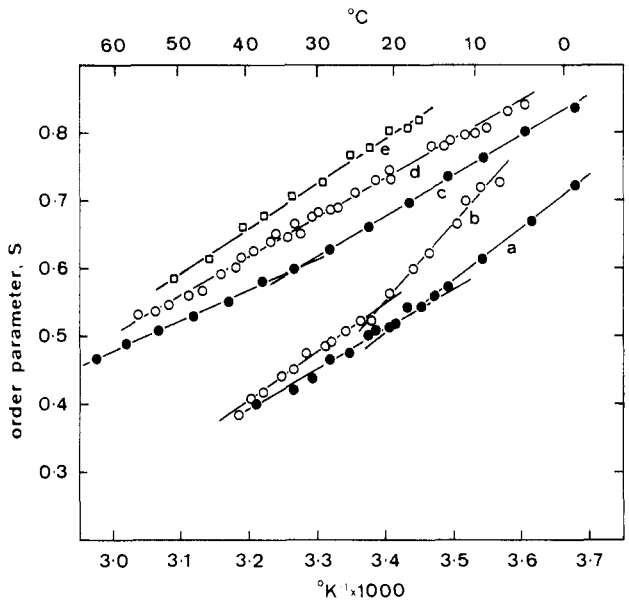

FIGURE 3: Order parameter S as a function of $1/T$ (K^{-1}) for (a and b) 12-doxylstearic acid incorporated into brush border vesicle membranes (b, O) and liposomes made from their extracted lipids (a, ●), (c and d) 5-doxylstearic acid in brush border vesicle membranes (d, O) and the liposomes (c, ●), and (e) 5-doxyl-PC incorporated into brush border vesicle membranes (□). Experimental conditions are given in the legend to Figure 1 for (a–d) and in the legend to Figure 2 for (e).

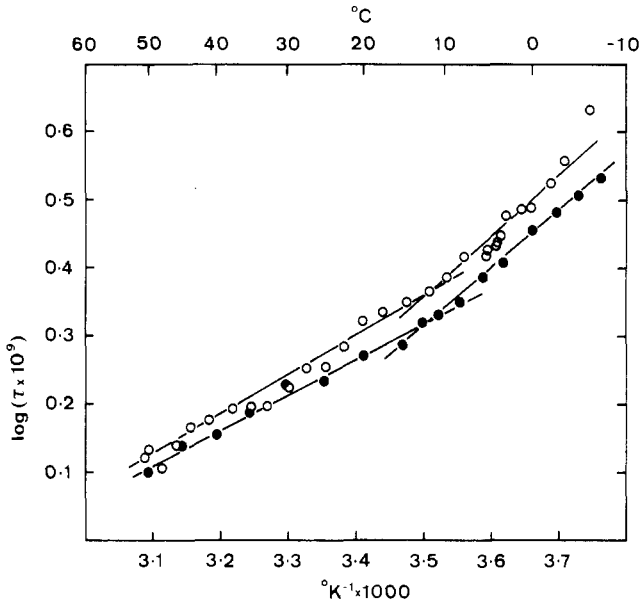

FIGURE 4: Arrhenius plot of rotational correlation time (τ) for 16-doxylstearic acid incorporated into brush border vesicle membranes (O) and liposomes (●) made from their extracted lipids. Experimental conditions are given in the legend to Figure 1.

The a_N values of 5-doxylstearic acid and the phospholipid label are between that measured in H_2O, a_N = 15.6 G, and that measured in ethanol, a_N = 14.6 G [Seelig & Hasselbach, 1971; cf. Dodd et al. (1970)], while the a_N values for 12- and 16-doxylstearic acid are representative of an apolar environment.

In Figure 3, the order parameter S, calculated according to eq 1, is shown as a function of $1/T$. The lower curves (a and b) are for the 12-doxylstearic acid incorporated into brush border vesicles (b) and the liposomes (a). Over the whole temperature range monitored, S is higher for the spin-label incorporated into brush border vesicles than into the liposomes. These plots (Figure 3a,b) both consist of two apparently straight line sections with discontinuity at about 18 °C for the liposomes and at about 22 °C for brush border vesicles. Essentially similar results are found for 5-doxylstearate acid (Figure 3c,d) except that when this spin-label is incorporated into brush border vesicles the data appear to lie on a continuous straight line. The data for the same probe incorporated into the liposomes appear to lie on two straight lines with a discontinuity at about 33 °C. 5-Doxylstearic acid was used to test different brush border preparations under different conditions. Brush border vesicles prepared by the standard technique (Schmitz et al., 1973; Kessler et al., 1978) with $CaCl_2$ as the precipitating agent and then labeled with 5-deoxylstearic acid gave results consistent with the data shown in Figure 3c,d. Over the temperature range tested (0–40 °C), S agreed within experimental error with the S values shown in Figure 3 for brush border vesicles prepared in the presence of EGTA and by using $MgCl_2$ as the precipitating agent. In both kinds of preparation, S was not affected appreciably when either 1 mM $CaCl_2$ or 10 mM EDTA was added to the buffer.

Figure 3e shows the temperature dependence of the order parameter S when 5-doxyl-PC was incorporated into brush border vesicles. The data appear to lie on a single straight line over the temperature range for which S could be measured. At temperatures <15 °C, line broadening did not allow the determination of $2T_\perp$ with sufficient accuracy to calculate S.

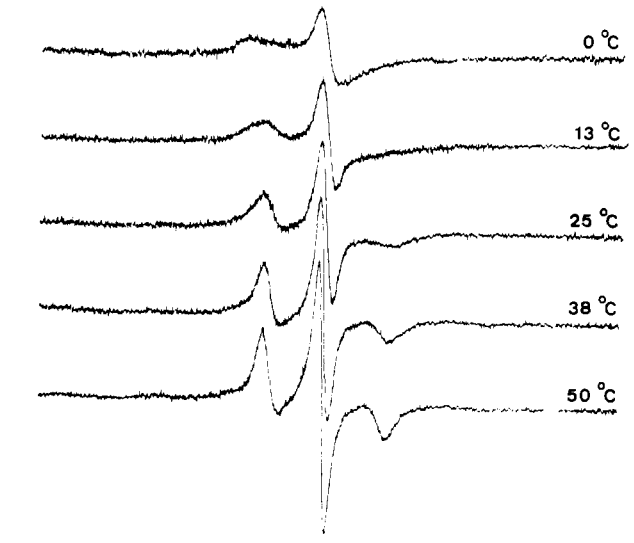

FIGURE 5: Temperature dependence of the ESR spectra of the cholestane spin-label incorporated into brush border vesicle membranes dispersed in the same buffer as in Figure 1 and at 29 mg of protein/mL (~16 mg of lipid/mL). The lipid:spin-label molar ratio was 100:1.

If, however, the maximum hyperfine splitting $2T_\parallel$ is plotted as a function of $1/T$, the data (not shown) are best fitted by two straight lines intersecting at about 18 °C. In contrast, the same plot for 5-doxylstearic acid gives a straight line (data not shown).

Comparison of the spectra in parts A and B of Figure 1 shows that the line width of 16-doxylstearic acid incorporated into brush border vesicles or the liposomes clearly increases with decreasing temperature. In Figure 4, log τ (τ = rotational correlation time) for 16-doxylstearic acid is plotted as a function of $1/T$. At any given temperature, the τ value is greater in brush border vesicle membranes than in the liposomes. Both plots appear to consist of two reasonably straight line sections with a discontinuity at about 13 °C.

The temperature dependence of the spectra of the cholestane spin-label incorporated into brush border vesicles is shown in Figure 5. At a given temperature, the spectral shapes are very similar to those for the same label incorporated into liposomes

Table III: Order Parameters and Correlation Times for Spin-Labeled Fatty Acids Incorporated into Various Cell Membranes and Lipids Extracted from These Membranes[a]

membrane	growth or body temp (°C)	S 5-doxylstearic acid	S 12-doxylstearic acid	$\tau \times 10^{10}$ (s), 16-doxylstearic acid	ref
rabbit small intestinal brush border vesicles	37	0.60	0.38	20.2	this work
liposomes from above	37	0.55	0.37	17.5	this work
Tetrahymena pyriformis					Iida et al. (1978)
microsomes	15		0.56		
pellicle	15		0.54		
cilia	15		0.63		
microsomes	40		0.61		
pellicle	40		0.61		
cilia	40		0.68		
Halobacterium cutirubrum	37	0.71	0.62	15	Esser & Lanyi (1973)
liposomes from above	37	0.62	0.46	10	Plachy et al. (1974)
Chinese hamster ovary	37	0.53			Lai et al. (1980)
chromaffin granule membrane from cow	37	0.57	0.37		Fretten et al. (1980)
liposomes from above	37	0.55	0.31		
plasma membrane of sarcoma 180 mouse ascites tumor	37	0.55	0.33		Bales et al. (1977)
egg phosphatidylcholine	37	0.56			Gordon & Sauerheber (1977)

[a] Parameters were measured at 37 °C.

made from lipids extracted from brush border vesicles (data not shown). The ESR spectra (Figure 5) indicate anisotropic motion of the steroid nucleus. The maximum hyperfine splittings $2T_\perp$ increase from ≃41 G at 50 °C to ≃44 G at 25 °C and show a discontinuity between 10 and 20 °C; below this temperature region, the spectral shape approaches that of a powder, and $2T_\perp$ increases to 62–64 G, i.e., $T_\perp \rightarrow T_{zz}$.

Discussion

^{31}P NMR spectra from rabbit small intestinal brush border vesicles recorded over a temperature range of 0–45 °C and under conditions comparable to those used in this study are typical of lipids in the lamellar phase (data not shown; L. Barsukov and H. Hauser, unpublished results). The ESR spectra of 5- and 12-doxylstearic acid and 5-doxyl-PC both in brush border vesicles and in the liposomes made from their extracted lipids are characteristic of rapid anisotropic motion. Given that the lipids together with the spin-labels are present in bilayers, the ESR results are consistent both with a preferred orientation of the above spin-labels in which the molecular long axis of the label is essentially perpendicular to the bilayer surface and with rapid rotation of the spin-label about this long axis, i.e., the bilayer normal (Hubbell & McConnell, 1969, 1971). It is reasonable to assume, as other investigators have done [e.g., see Seelig (1976)], that to a first approximation the motion of the spin probe is representative of the average motion of a lipid molecule.

Results obtained with the cholestane spin-label are consistent with these conclusions. At temperatures >20 °C, the maximum hyperfine splitting $2T_\perp$ is of the order of 40 G. This value is interpreted as the result of rapid motion of the steroid nucleus about its long axis which averages out the hyperfine splitting tensor components, T_{zz} and T_{xx}, to give $2T_\perp = T_{zz} + T_{xx}$ and $T_\parallel = T_{yy}$ (Hubbell & McConnell, 1969; Hsai et al., 1970; Knowles et al., 1976). When the temperature is decreased below 10–20 °C, the observation that $2T_\perp$ is greater than 60 G and approaches $2T_{zz}$ indicates that under these conditions the rotational motion about the long axis of the molecule is frozen.

A decrease in the order (packing) of the membrane with increasing temperature is demonstrated by the temperature dependence of the ESR spectra of the 5- and 12-doxylstearic acids, the 5-doxyl-PC, and the cholestane spin-label (Figures 1, 2, and 5); this is also demonstrated by the temperature dependence of the S value of the first three of these probes (Figure 3). Consistent with this finding is the temperature-dependent increase in Tempo distribution (Figure 6) and in fluidity (microviscosity) as monitored by 16-doxylstearic acid. Further, the motion of the spin probes increases with increasing depth in the membrane or lipid bilayer. Both these fluidity gradients are to be expected and have been found previously for both lipid bilayers and biological membranes (Rottem et al., 1970; Hubbell & McConnell, 1971; Jost et al., 1971; Seelig, 1971; Levine et al., 1972; Seelig & Seelig, 1974; Devaux et al., 1975).

In Table III, the order parameters of the 5- and 12-doxylstearic acids and the rotational correlation time τ of 16-doxylstearic acid found in this work are compared with values taken from the literature. This comparison has been made at 37 °C which is, in most cases, the growth or body temperature of the organism. In general, there are appreciable differences between mammalian and prokaryote cell membranes, but there is less variation between different mammalian cell membranes. For the mammalian membranes, the order parameter S of 5-doxylstearic acid lies between 0.53 and 0.60, the S value for the same label in egg yolk PC being in the middle of this range. The value for brush border vesicles is at the top end of this range but is considerably lower than the value for the halophilic *Halobacterium cutirubrum*. The order parameter of the 12-doxylstearic acid in brush border vesicles is very similar to that in the chromaffin granule membrane.

The rotational correlation time of 16-doxylstearic acid is somewhat longer in the brush border vesicle membrane than in *Halobacterium cutirubrum* (Table III), indicating that the microviscosity of the hydrophobic region of the brush border vesicle membrane and of the liposomes is relatively high. As regards the order (packing) and the microviscosity, the conclusions drawn from our spin-label work are qualitatively in good agreement with those of Schacter & Shinitzky (1977) and Brasitus et al. (1980), who used the polarization of the fluorochrome diphenylhexatriene to measure the microviscosity of brush border vesicle membranes from rat small intestine.

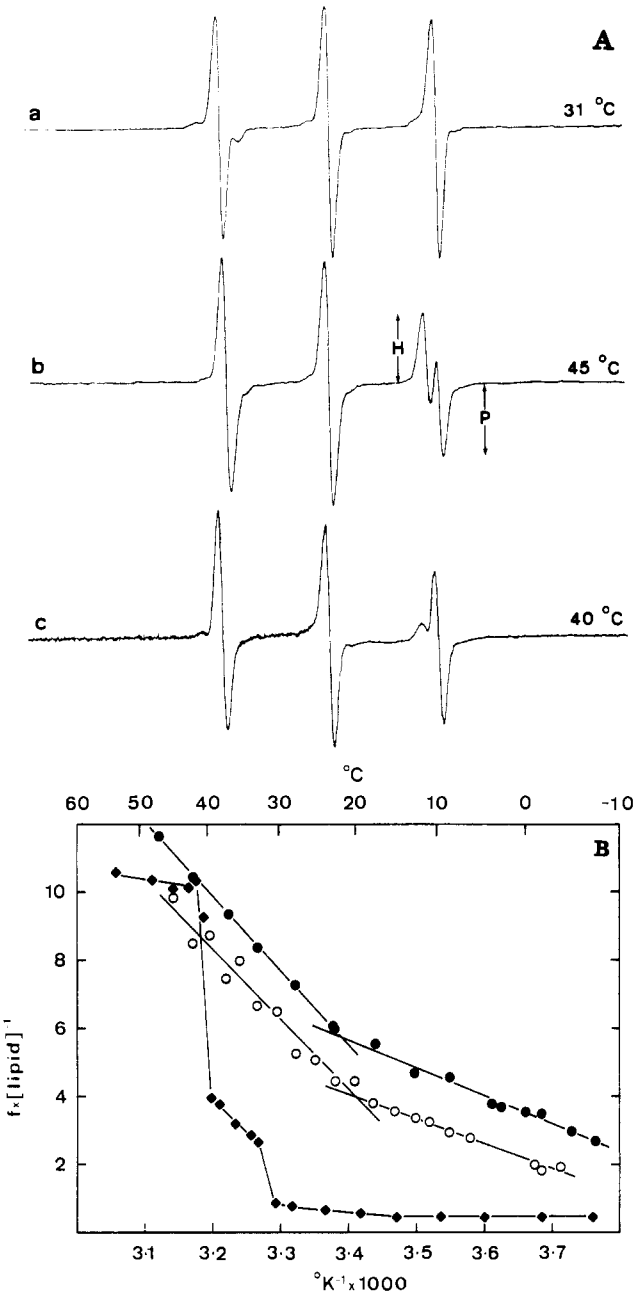

FIGURE 6: (A) ESR spectra of (a and b) Tempo (10 μM) added to dipalmitoyl-PC (46 mg/mL, 60 mM) dispersed in water and (c) Tempo (100 μM) added to brush border vesicles (39 mg of protein/mL, ~21 mg of lipid/mL) in the same buffer as in Figure 1. The temperature in (a) is below, and that in (b) above, the gel to liquid-crystalline transition of dipalmitoyl-PC. (B) Tempo partitioning into brush border vesicle membranes (○), into liposomes made from their extracted lipids (●), and into liposomes made from dipalmitoyl-PC (♦) as a function of $1/T$. Lipids extracted from brush border vesicles were dispersed at 9.2 mg/mL in buffer (the same as in Figure 1) containing 100 μM Tempo; other experimental details are as in (A). The spectral parameter f has been divided by the lipid concentration in grams per milliliter.

These authors also concluded that the lipid fluidity is relatively low (i.e., the microviscosity is relatively high) compared with that of other mammalian membranes.

It is clear from Figure 3 that the proteins associated with brush border vesicle membranes increase the order parameter monitored by the spin-labels, i.e., the rigidity or packing order of the membrane, compared to the liposomes made from their extracted lipids. This effect of protein has been found for other membranes (Rottem et al., 1970; Tourtellotte et al., 1970; Raison et al., 1971). Consistent with this result are the observations that the rotational correlation time τ of 16-doxylstearic acid is longer in brush border membrane vesicle membranes than in the liposomes (Figure 4) and that the Tempo distribution coefficient is smaller in brush border membrane vesicles than in the liposomes (Figure 6B); these latter results indicate that in the absence of protein the lipid fluidity is increased. A similar effect was reported for rat brush border vesicle membranes (Schacter & Shinitzky, 1977; Brasitus et al., 1980) except that a larger increase in lipid fluidity was observed in the absence of membrane proteins. An alternative interpretation of the Tempo distribution is that proteins sequester lipids out of the bulk phase, thus effectively reducing the total amount of lipid accessible to Tempo (Jost et al., 1973; Warren et al., 1975; Curatolo et al., 1978; Chapman et al., 1979; Davoust et al., 1980).

Table IV: Temperatures at Which Discontinuities Were Observed in Various Temperature-Dependent ESR Parameters of the Spin-Labels[a]

spin-label	ESR parameter	temp of discontinuity (°C) vesicles	liposomes
5-doxyl-PC	S	no break	ND[b]
	$T_{\parallel}$	18	ND
	a_N	18–20	ND
5-doxylstearic acid	S	no break	30
	$T_{\parallel}$	no break	26
	a_N	25	25
12-doxylstearic acid	S	22	18
	$T_{\parallel}$	21	18
	a_N	23	no break
16-doxylstearic acid	τ	13	13
	a_N	no break	no break
cholestane label	$T_{\parallel}$	20	20
Tempo	f	18–20	18–20

[a] Parameters were monitored over a temperature range of at least 5–40 °C. The spin-labels were incorporated into brush border vesicle membranes and into liposomes made from their extracted lipids. [b] ND, not determined.

The temperature dependence of S (Figure 3), of Tempo distribution (Figure 6B), and of the rotational correlation time τ (Figure 4) shows a discontinuity that is at a different temperature for each of the probes and lies between 13 and 30 °C (Table IV). A discontinuity was also observed in the temperature dependence of the hyperfine splitting constant a_N of 5- and 12-doxylstearic acid and of 5-doxyl-PC and in the temperature dependence of $2T_{\perp}$ of the cholestane spin-label. These data are summarized in Table IV. This shows that where a discontinuity can be detected in both the brush border vesicle membranes and the liposomes, it occurs at a similar temperature, suggesting that the underlying cause of the discontinuities arises from some intrinsic property of the membrane lipids that is not greatly influenced by the presence of membrane protein. The fact that the discontinuities are observed with various spin-labels differing widely in their chemical nature and that they are observed in the temperature dependence of different ESR parameters suggests that the discontinuity represents a lipid phase transition and/or phase separation. Direct evidence for such a lipid phase change cannot be derived from the ESR data presented here. Differential scanning calorimetry could shed light on this question. Preliminary experiments show that the lipids of brush border vesicle membranes undergo a broad, reversible thermotropic phase transition (H. Hauser, unpublished results). The discontinuities observed in the temperature dependence of various ESR parameters are, therefore, tentatively assigned to thermotropic lipid phase transitions and/or phase separations. Previous ESR spin-label studies have shown that breaks in the

temperature dependence of ESR parameters (e.g., correlation time, τ) correlate well with breaks in the Arrhenius plot of membrane protein activities; this has been observed in the temperature range 18–25 °C in rat liver mitochondria (Williams et al., 1972; Tinberg et al., 1972), in rat liver microsomes (Eletr et al., 1973), and in sarcoplasmic reticulum (Eletr & Inesi, 1972).

Inspection of Table IV shows that the deeper the spin-label is in the membrane the lower the temperature at which the discontinuity occurs. These values are not particularly exact, but they are quite distinct from one another. One possible interpretation of this finding is a progressive melting of the hydrocarbon chains starting, at about 10 °C, in the middle of the lipid bilayer and subsequently moving along the hydrocarbon chains toward the surface of the membrane. Such an interpretation is supported by NMR and ESR data (Seelig & Seelig, 1974; Jost et al., 1971; Fretten et al., 1980).

Our finding of a broad reversible phase transition is similar to results reported for rat brush border vesicle membranes (Schacter & Shinitzky, 1977; Brasitus et al., 1980). Using differential scanning calorimetry, these authors were able to demonstrate reversible thermotropic lipid phase transitions in both intact membranes and dispersions of lipids extracted from them. In both cases, broad transitions were observed between 23 and 39 °C, with the peak temperature at about 30 °C. Compared to our data for rabbit small intestinal brush border vesicles, these transitions appear to be about 10 °C higher. This difference probably reflects differences in the lipid composition of these membranes.

Unfortunately, very little is known about the structure and organization of brush border vesicle membranes, and a discussion of the functional significance of the high order of packing and of the relatively low microviscosity of the hydrophobic lipid region of brush border vesicle membranes would only be speculative. However, it is safe to say that the lipid phase transition cannot play a physiological role since the transition temperature is well separated from body temperature. Further, some degree of lipid fluidity, as measured at 37 °C, is apparently required for optimum activity of transport proteins and enzymes that are intimately associated with the membrane lipids [cf. Brasitus et al. (1979)]. Nothing can be derived from our ESR data concerning the lateral distribution (i.e., in the plane of the membrane) of the lipids and proteins of the brush border vesicle membrane or the effect of temperature on this distribution. Hence, nothing can be said about the molecular mechanism underlying the phase transition which might involve the crystallization of lipid hydrocarbon chains, clustering of certain lipids, or other unknown mechanisms. The ESR results obtained with the cholestane spin-label at temperatures below the transition range are consistent with a crystalline hydrocarbon chain lattice where the rotational averaging about the molecular long axis has stopped.

Acknowledgments

We thank I. Püttner, R. Renetseder, and B. Mütsch for their assistance.

References

Bales, B. L., Lesin, E. S., & Oppenheimer, S. B. (1977) *Biochim. Biophys. Acta 465*, 400–407.

Barsukov, L. I., Hauser, H., Hasselbach, H.-J., & Semenza, G. (1980) *FEBS Lett. 115*, 189–192.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–252.

Brasitus, T. A., & Schacter, D. (1980) *Biochemistry 19*, 2736–2768.

Brasitus, T. A., Schacter, D., & Mamonneas, T. G. (1979) *Biochemistry 18*, 4136–4144.

Brasitus, T. A., Tall, A. R., & Schacter, D. (1980) *Biochemistry 19*, 1256–1261.

Brunner, J., Hauser, H., & Semenza, G. (1978) *J. Biol. Chem. 253*, 7538–7546.

Chakrabarti, P., & Khorana, H. G. (1975) *Biochemistry 14*, 5021–5033.

Chapman, D., Gomêz-Fernândez, J. C., & Gõni, F. M. (1979) *FEBS Lett. 98*, 211–233.

Curatolo, W., Verma, S. P., Sakura, J. P., Small, D. M., Shipley, G. G., & Wallach, D. F. H. (1978) *Biochemistry 17*, 1802–1807.

Davoust, J., Bienvenue, A., Fellman, P., & Devaux, P. F. (1980) *Biochim. Biophys. Acta 596*, 28–42.

Devaux, P., Bienvenüe, A., Lanquin, G., Brisson, A. D., Vignas, P. M., & Vignais, P. V. (1975) *Biochemistry 14*, 1272–1280.

Dodd, G. H., Barrat, M. D., & Rayner, L. (1970) *FEBS Lett. 8*, 286–288.

Eletr, S., & Inesi, G. (1972) *Biochim. Biophys. Acta 290*, 178–185.

Eletr, S., Zakin, D., & Vessey, D. A. (1973) *J. Mol. Biol. 78*, 351–362.

Esser, A. F., & Lanyi, J. K. (1973) *Biochemistry 12*, 1933–1939.

Folch, J., Lees, M., & Stanley, G. H. S. (1957) *J. Biol. Chem. 226*, 497–509.

Fretten, P., Morris, S. J., Watts, A., & Marsh, D. (1980) *Biochim. Biophys. Acta 598*, 247–259.

Gains, N., & Hauser, H. (1981) *Biochim. Biophys. Acta 646*, 211–217.

Gordon, L. M., & Sauerheber, R. D. (1977) *Biochim. Biophys. Acta 466*, 34–43.

Griffith, O. H., Dehlinger, P. J., & Van, S. P. (1974) *J. Membr. Biol. 15*, 159–192.

Gupta, C. M., Radhakrishnan, R., & Khorana, H. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4315–4319.

Hauser, H., Howell, K., Dawson, R. M. C., & Bowyer, D. E. (1980) *Biochim. Biophys. Acta 602*, 567–577.

Hsai, J. C., Schneider, H., & Smith, I. P. C. (1970) *Biochim. Biophys. Acta 202*, 399–402.

Hubbell, W. L., & McConnell, H. M. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 12–16.

Hubbell, W. L., & McConnell, H. M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 20–27.

Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314–326.

Iida, H., Maeda, T., Ohki, K., Nozawa, Y., & Ohnishi, S. I. (1978) *Biochim. Biophys. Acta 508*, 55–64.

Jost, P., Libertini, L. J., Herbert, V. C., & Griffith, O. H. (1971) *J. Mol. Biol. 59*, 77–98.

Jost, P. C., Griffith, O. H., Capaldi, R. A., & van der Kooi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 480–484.

Kamp, H. H., Wirtz, K. W. A., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 318*, 313–325.

Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M., & Semenza, G. (1978) *Biochim. Biophys. Acta 501*, 136–154.

Kivelson, D. (1960) *J. Chem. Phys. 33*, 1094–1106.

Knowles, P. F., Marsh, D., & Rattle, H. W. E. (1976) *Magnetic Resonance of Biomolecules*, p 266, Wiley, London.

Lai, C. S., Hopwood, L. E., & Swartz, H. M. (1980) *Biochim. Biophys. Acta 602*, 117–126.

Levine, Y. K., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1972) *Biochemistry 11*, 1416-1421.
Paltauf, F., Hauser, H., & Phillips, M. C. (1971) *Biochim. Biophys. Acta 249*, 539-547.
Plachy, W. Z., Lanyi, J. K., & Kates, M. (1974) *Biochemistry 13*, 4906-4913.
Raison, J. K., Lyons, J. M., Melhorn, R. J., & Keith, A. D. (1971) *J. Biol. Chem. 246*, 4036-4040.
Rottem, S., Hubbell, W. L., Hayflick, L., & McConnell, H. M. (1970) *Biochim. Biophys. Acta 219*, 104-113.
Schacter, D., & Shinitzky, M. (1977) *J. Clin. Invest. 59*, 536-548.
Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B. K., Cerda, J. J., & Crane, R. K. (1973) *Biochim. Biophys. Acta 323*, 98-112.
Seelig, A., & Seelig, J. (1974) *Biochemistry 13*, 4839-4845.
Seelig, J. (1971) *J. Am. Chem. Soc. 93*, 5017-5022.
Seelig, J. (1976) in *Spin Labeling Theory and Application* (Berliner, L. J., Ed.) pp 373-409, Academic Press, New York.
Seelig, J., & Hasselbach, W. (1971) *Eur. J. Biochem. 21*, 17-21.
Shimshick, E. J., & McConnell, H. M. (1973) *Biochemistry 12*, 2531-2360.
Stone, T. J., Buckman, T., Nordio, P. L., & McConnell, H. M. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1010-1017.
Tinberg, H. M., Packer, L., & Keith, A. D. (1973) *Biochim. Biophys. Acta 283*, 193-205.
Tomkiewicz, M., & Corker, G. A. (1975) *Biochim. Biophys. Acta 406*, 197-205.
Tourtellotte, M. E., Branton, D., & Keith, A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 909-916.
Warren, G. B., Houslay, M. D., Metcalfe, J. C., & Birdsall, N. J. M. (1975) *Nature (London) 255*, 684-687.
Williams, M. A., Stancliff, R. C., Packer, L., &. Keith, A. D. (1972) *Biochim. Biophys. Acta* 267, 444-456.

Specific Binding of Toxin II from *Centruroides suffusus suffusus* to the Sodium Channel in Electroplaque Membranes†

Kenneth P. Wheeler,‡ Jacques Barhanin, and Michel Lazdunski*

ABSTRACT: The binding of toxin II from the scorpion *Centruroides suffusus suffusus* (Css_{II}) to electroplaque membranes from *Electrophorus electricus* was studied with the use of a radiolabeled derivative of the toxin ($[^{125}I]Css_{II}$). Specific binding of the latter to the membranes required the protonation of a group, either in the membrane or in the toxin itself, with an apparent pK_a value of 7.5 and also the presence of a certain minimum concentration of ions, though there was no requirement for a specific ion. At 20 °C and pH 6 the second-order rate constant for formation of the $[^{125}I]Css_{II}$–membrane complex was about 5×10^6 M^{-1} s^{-1}, while the first-order constant for its dissociation was about 2×10^{-3} s^{-1}. Under equilibrium conditions specific binding of $[^{125}I]Css_{II}$ was a simple saturable function of $[^{125}I]Css_{II}$ concentration, characterized by a dissociation constant of 0.4–0.7 nM and a maximum capacity of 0.9–2.4 pmol of toxin/mg of membrane protein. The latter value was the same as the number of membrane sites that could specifically bind a radiolabeled derivative of tetrodotoxin. Unlabeled Css_{II} displaced bound $[^{125}I]Css_{II}$ with an apparent dissociation constant of about 1 nM. None of 19 other neurotoxins or local anaesthetics known to interact with Na^+ channels in excitable cells affected $[^{125}I]Css_{II}$ binding, but it was completely inhibited by toxin γ from the scorpion *Tityus serrulatus serrulatus*. These findings suggest that the Na^+ channel possesses a distinct class of binding sites to which these two scorpion toxins bind with high affinities. On the other hand, no Css_{II} receptor was detected in crab axonal membranes, indicating that it is not a characteristic feature of all Na^+ channels.

The primary importance of the Na^+ channel in the functioning of excitable cells has been emphasized by the discovery that a considerable number of naturally occurring toxins exert their effects by reacting specifically with the Na^+ channel, causing inhibition or modification of its normal behavior. Moreover, it is now clear that such neurotoxins do not all act on the Na^+ channel in the same way. Combined biochemical and electrophysiological studies have revealed the existence of a number of distinct binding sites for different toxins, together with at least four characteristically different ways of interference with the Na^+ channel's functioning (Narahashi, 1974; Ritchie & Rogart, 1977; Catterall, 1980; Howard & Gunderson, 1980; Lazdunski et al., 1980a; Rogart, 1981; Honerjäger, 1981). These latter findings are particularly important because they show how these neurotoxins can be used as specific probes for investigations at the molecular level of the structure, function, and differentiation of the Na^+ channel (Levinson & Ellory, 1973; Agnew et al., 1978; Barchi et al., 1980; Lazdunski et al., 1980b; Armstrong, 1981; Lazdunski & Renaud, 1982).

The fact that some of these neurotoxins interact with the Na^+ channel in different ways is not surprising in view of their radically different chemical compositions and molecular structures. But recent findings have indicated that some neurotoxins of apparently very similar composition and structure also produce characteristically different modifications of Na^+ channel functioning. In particular, the polypeptide toxin II extracted from the venom of the Mexican scorpion *Centruroides suffusus suffusus* has been found to act very differently from apparently similar polypeptide toxins derived

† From the Centre de Biochimie du CNRS, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France. *Received February 18, 1982.* This work was supported by Délégation Générale à la Recherche Scientifique et Technique Grant 81.E.0575, l'Institut National de la Santé et de la Recherche Médicale Grant 80.30.10, and la Foundation pour la Recherche Médicale et le Centre National de la Recherche Scientifique.

‡ On leave from the School of Biological Sciences, University of Sussex, Brighton BN1 9QG, U.K.

0006-2960/82/0421-5628$01.25/0