Order–Disorder Phase Transition and Lipid Dynamics in Rabbit Small Intestinal Brush Border Membranes. Effect of Proteins†

Beat Mütsch, Nigel Gains, and Helmut Hauser*

ABSTRACT: The total lipids extracted from brush border membranes form smectic lamellar phases when dispersed in water. ^{31}P broad-band nuclear magnetic resonance (NMR) shows that between body temperature (37 °C) and freezing of the solvent, the extracted lipids form bilayers with the lipid molecules undergoing fast anisotropic motion. This is also true for the lipids present in the brush border membrane. The electron spin resonance (ESR) results obtained with various hydrophobic spin probes incorporated in either brush border vesicle membranes or their extracted lipids are consistent with this interpretation. By use of a variety of chemically different spin-labels, the temperature dependence of brush border membranes and their extracted lipids was probed. The temperature dependence of various ESR spectral parameters shows discontinuities that, by comparison with differential scanning calorimetry, are assigned to a lipid thermotropic phase transition. Differential scanning calorimetry shows that the lipid in brush border membranes undergoes a broad, reversible phase transition of low enthalpy between 10 and 30 °C, with a peak temperature of about 25 °C. Hence, the brush border membrane of rabbit small intestine functions in the liquid-crystalline state, well above the peak temperature and also above the upper limit of the lipid phase transition. Therefore, in itself, the thermotropic lipid phase transition is unlikely to play a physiological role. The low enthalpy of the lipid phase transition, indicative of a lack of cooperativity, is primarily attributed to the relatively high cholesterol content and to heterogeneity in the lipid composition of this membrane [Hauser, H., Howell, K., Dawson, R. M. C., & Bowyer, D. E. (1980) *Biochim. Biophys. Acta 602*, 567–577]. Lipid–protein interaction seems to play a minor role in this context. Papain treatment and also alkaline treatment of brush border vesicles produce significant protein losses. Papain digestion solubilizes about 65% of the total protein; alkaline treatment at pH 8.8 and 37 °C and at pH 11 and 4 °C leads to protein losses of 60 and 58%, respectively. The alkaline treatment at pH 11 leads to the quantitative removal of the cytoskeletal proteins. Removal of these membrane-bound enzymes and of the cytoskeletal proteins apparently has no effect on the fluidity and molecular packing of brush border vesicles. It also does not affect the reversible thermal behavior of these vesicles.

The microvillus plasma membrane or brush border membrane of the small intestine is highly specialized for the digestion and absorption of nutrients. Previous studies of brush border membrane from rat small intestine by fluorescence polarization (Schachter & Shinitzky, 1977; Brasitus et al., 1980) and from rabbit small intestine with a spin-label approach (Hauser et al., 1982) have shown that this plasma membrane is characterized by a relatively high lipid "microviscosity" and packing order compared to other mammalian plasma membranes. By comparing the microviscosity and order parameters measured in brush border membranes with the same parameters measured in liposomes made from the lipid extract of this membrane, it was concluded that the membrane fluidity is primarily determined by the lipid composition and not significantly affected by the presence of membrane protein (Schachter & Shinitzky, 1977; Brasitus et al., 1980; Hauser et al., 1982). The same conclusion could be drawn from the comparison of the thermal behavior of brush border membrane with that of liposomes made from the lipid extract (Schachter & Shinitzky, 1977; Brasitus et al., 1980; Hauser et al., 1982). As reported, discontinuities were observed in the temperature dependence of various ESR[1] spectral parameters for brush border membrane, and similar discontinuities were observed for the lipid extract of this membrane. These discontinuities were tentatively assigned to order–disorder lipid phase transitions. Here, we show by differential scanning calorimetry that they are indeed due to reversible broad lipid phase transitions. Furthermore, it is shown that removal of a considerable proportion of membrane-bound enzymes and the quantitative removal of the cytoskeleton have no significant effect on the packing and fluidity of the brush border membrane. Removal of these proteins, which can amount to about 70% of the total protein of brush border vesicles, has no significant effect on the thermal behavior of the lipid part of the membrane.

Materials and Methods

The spin-labeled phosphatidylcholines 1-palmitoyl-2-(5-doxylstearoyl)-3-*sn*-phosphatidylcholine (5-doxyl-PC) and 1-palmitoyl-2-(8-doxylpalmitoyl)-3-*sn*-phosphatidylcholine (8-doxyl-PC) were prepared from 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (purchased from Berchtold, Biochemisches Labor, Bern) as described previously (Hauser et al., 1982). The stearic acid spin-labels with the doxyl group attached to either C-5 or -16 and the cholestane spin-label were purchased from Syva (Palo Alto, CA). Papain was obtained from Boehringer (Mannheim, FRG).

Brush border membrane vesicles[2] were prepared from rabbit small intestine stored at −80 °C according to Schmitz et al. [1973; cf. Kessler et al. (1978) and Gains & Hauser (1981)]. However, the procedure was modified in that contaminating

† From the Laboratorium für Biochemie, Eidgenössische Technische Hochschule Zürich, ETH-Zentrum, CH 8092 Zürich, Switzerland. *Received May 17, 1983.* This work was supported by the Swiss National Science Foundation (Grant 3.156-0.81).

[1] Abbreviations: ESR, electron spin resonance; DEPBa, barium salt of diethyl phosphate, $Ba[(C_2H_5)_2PO_4]_2$; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid disodium salt; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; TLC, thin-layer chromatography; Tris tris(hydroxymethyl)aminomethane.

[2] The brush border membranes isolated from rabbit small intestine according to the method used here have been described as consisting essentially of closed membrane vesicles (Kessler et al., 1978); they will be referred to in this paper as brush border vesicles.

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membranes, dispersed in 300 mM D-mannitol–10 mM Hepes plus Tris to pH 7.0 to 7.5, were precipitated with 10 mM $MgCl_2$ (Hauser et al., 1980). Lipids were extracted from brush border vesicles essentially as described before (Hauser et al., 1982) except that 5 mM EDTA was added to the brush border vesicle dispersion before addition of the organic solvent. The presence of EDTA is necessary if lipolysis during the lipid extraction is to be minimized.

The fatty acid and cholestane spin-labels were incorporated into brush border vesicles as described before (Hauser et al., 1982). Liposomes were made from the lipid extract and labeled with the cholestane spin probe as described in this reference (Hauser et al., 1982). Spin-labeled PC was incorporated into brush border vesicle membranes with PC exchange protein by the procedure of Barsukov et al. [(1980); see also Hauser et al. (1982)].

Various Treatments of Brush Border Vesicles. An increase in pH of the dispersion medium leads to the release of protein from brush border vesicles. Brush border vesicles were suspended in 10 mM Tris-HCl, pH 8.8, containing 5 mM EDTA, 300 mM D-mannitol, and 0.02% sodium azide at 4 °C and pelleted by centrifugation at 4 °C and 38000*g* for 30 min. By this treatment, ~30% of the protein was lost as determined by protein analysis according to Lowry et al. (1951; bovine serum albumin was used as a standard); hence, the phospholipid to protein ratio increased from about 12 (before treatment) to 18 μg of P/mg of protein. The pellet of brush border vesicles was resuspended in the same Tris buffer, pH 8.8, and brush border vesicles were incubated at 37 °C for 60 min. After incubation, the brush border vesicles were pelleted by two centrifugations at 60000*g* for 30 min. Protein analysis of the pellet showed that another 30% of the original total membrane protein was released. The lipid of the pellet was extracted as described above and analyzed for phosphorus according to Chen et al. (1956). This analysis showed that lipid was also lost during this incubation; since lipid and protein were lost in the same ratio as found in brush border vesicles before incubation, the phospholipid to protein ratio of 18 μg of P/mg of protein was not significantly changed. This suggests that the incubation of brush border vesicles at pH 8.8 and 37 °C for 60 min leads to structural changes in at least some of the brush border vesicles, which, as a consequence, are no longer precipitable at 60000*g*.

Brush border vesicles were digested with papain at 37 °C for 45 min (Sigrist et al., 1975). Papain and proteins that were hydrolyzed and released from the brush border membrane were separated from brush border vesicles by column chromatography on Bio-Gel A-5m. The effect of the proteolytic digestion on the protein composition of brush border vesicles was monitored by protein analysis and by sodium dodecyl sulfate–PAGE (Laemmli, 1970). The protein loss amounted to about 65%, and the phospholipid loss was about 30%. Due to the proteolytic digestion and release of protein from brush border vesicles, the phospholipid to protein ratio increased from 13 to 23 μg of P/mg of protein.

Alkaline treatment of brush border vesicles at high pH led to the quantitative release of actin. Brush border vesicles, prepared as described above, were suspended at 0.3 mg of protein/mL in 300 mM D-mannitol–4 mM EDTA (tetrasodium salt) at pH 11 and at 4 °C for 15 min. This treatment released >95% of the actin (and also other presumably cytoskeletal or cytoplasmic proteins) into solution. The released proteins remained in solution when the pH was returned to pH 7.5. At this pH, the membrane fraction, which contained the membrane-associated protein sucrase/isomaltase (identified by sodium dodecyl sulfate–PAGE), was precipitated by centrifugation at 45000*g* for 30 min. Phospholipid and TLC analysis of brush border vesicles after alkaline treatment at pH 11 showed that these treatment did not lead to phospholipid degradation. No loss of phospholipid was detected.

D-Glucose transport in the presence of a Na^+ gradient and sucrase activity were determined in brush border vesicles according to Hopfer et al. (1973) and Banauch et al. (1975), respectively. The temperature dependence of the sucrase activity in brush border vesicles was measured between 0 and 37 °C and that of the D-glucose transport between 0 and 30 °C. The D-glucose transport was measured by determining the amount of D-glucose taken up by brush border vesicles after 3 s of incubation in the presence of a NaCl concentration gradient ($[NaCl]_{outside}$ = 0.1 M; $[NaCl]_{inside}$ = 0).

For ESR measurements, brush border vesicles (10–30 mg of protein/mL) were dispersed in buffer (10 mM Hepes/Tris, pH 7, 300 mM D-mannitol, 0.02% NaN_3), and the lipids (10–20 mg) extracted from brush broder vesicles were dispersed in the same buffer (Hauser et al., 1982). The lipid to spin-label molar ratio was >100. Samples, 20–50 μL, were measured in glass capillary tubes of 1-mm internal diameter. For ESR measurements, labeled brush border vesicles and liposomes made from the lipid extract were kept on ice, and all measurements were carried out during the course of 1 day. For differential scanning calorimetry (DSC), brush border vesicles dispersed in buffer (10 mM Hepes/Tris, pH 7.6, 300 mM D-mannitol) were centrifuged at 4 °C and 100000*g* for 1 h. The pellet was filled into the DSC pan (volume ~75 μL), which was sealed immediately and transferred to the DSC instrument. A chloroform–methanol solution (2:1 v/v) of the lipids extracted from brush border vesicles was taken to dryness by rotary evaporation. The lipids were dried in vacuo for 1 h and weighed into the DSC pan. After the appropriate amount of H_2O or buffer (as above) was added, the DSC pan was sealed and transferred to the DSC instrument. ESR spectra were recorded at 9.2 GHz with a Varian X-band spectrometer (Model E-104A) fitted with a variable-temperature control. The temperature was monitored with either a thin thermometer or a small thermocouple and was accurate to ±0.5 °C. Order parameters S and orientational correlation times τ_c were determined as described previously (Hauser et al., 1982). The calorimetric measurements were carried out in a Perkin-Elmer (Norwalk, CT) DSC-2 differential scanning calorimeter. Samples were heated and cooled repeatedly, usually at a rate of 2.5 °C/min. The instrument was calibrated by using gallium, indium, and cyclohexane as standards. Transition enthalpies were determined from the area under the peak as measured by planimetry. Proton-decoupled ^{31}P NMR spectra were recorded on a Bruker CXP 300 spectrometer operating at 121.47 MHz in the Fourier-transform mode. Spectra were accumulated by using the Hahn spin-echo pulse sequence, $(90_x–\tau–180_x–AQ–PD)_n$, with a 90° pulse of 3 μs, a refocusing time τ = 90 μs, an acquisition time AQ = 150 ms, and a pulse delay PD = 8–10 s. NMR spectra of membranes and lipid dispersions below the transition temperature were obtained by using the cross-polarization method.

Results

ESR. Figure 1 shows the temperature dependence of ESR spectra of the cholestane spin-label incorporated into liposomes made from the total lipid extract of brush border membranes. At a given temperature, the spectrum is very similar to that of the same label incorporated into brush border membranes (Hauser et al., 1982). The ESR spectra are characteristic of anisotropic motion of the steroid nucleus. The temperature

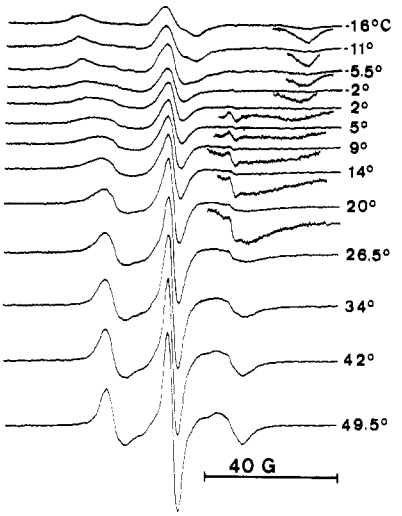

FIGURE 1: Temperature dependence of ESR spectra of cholestane spin-label incorporated into lipids extracted from brush border vesicles. The lipids were dispersed in buffer (10 mM Hepes/Tris, pH 7, 5 mM EDTA, 300 mM D-mannitol, 0.02% NaN_3) as described under Materials and Methods. For temperatures ≤20 °C, vertical expansion of the high-field part of the spectrum is shown.

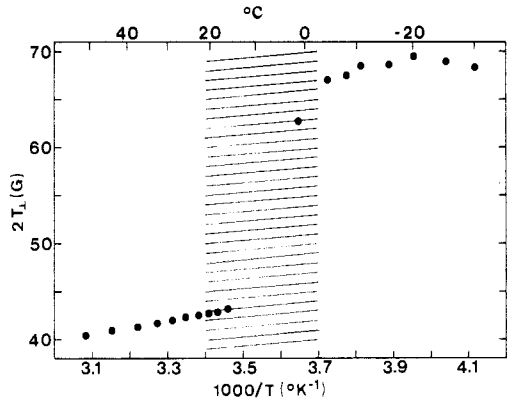

FIGURE 2: Maximum hyperfine splitting $2T_\perp$ (G) as a function of $1/T$ (K^{-1}); $2T_\perp$ values were derived from the ESR spectra of the cholestane spin-label shown in Figure 1. The hatched region between about 20 and −2 °C represents the transition from motional-averaged to immobilized spectra.

dependence of the maximum hyperfine splittings $2T_\perp$ is shown in Figure 2. The value of $2T_\perp$ increases from about 40 G at 50 °C to 43 G at 20 °C. At temperatures <0 °C, immobilized spectra are observed with $2T_\perp$ being greater than 60 G; under these conditions, $2T_\perp$ approaches $2T_{zz}$, indicating that the rotation about the long axis of the molecule is frozen. In the temperature range between 0 and 20 °C (hatched area), there is a transition between the motionally averaged spectra observed above ~20 °C and the immobilized spectra observed below 0 °C. In this transition range the ESR spectra (Figure 1) appear to be a superposition of several spectra differing in molecular motion.

The ESR spectra of 5-doxylstearic acid incorporated in brush border vesicle membranes are typical for lipid bilayers in which the label is undergoing rapid but anisotropic motion. As described previously (Hauser et al., 1982), at a given temperature the ESR spectra of 5-doxylstearic acid incorporated into brush border vesicle membranes are very similar to those for the same label in liposomes made from the lipid extract (data not shown). Figure 3 shows the temperature dependence of the order parameter S derived from the ESR spectra of 5-doxylstearic acid incorporated into brush border vesicle membranes (solid line). This is compared to the temperature dependence of S for the same label in brush border membranes incubated at pH 8.8 and 37 °C for 1 h (●) and after papain treatment of brush border vesicles (×). In all three cases, there is an approximately linear relationship between S and $1/T$. Although the order parameter of the treated brush border membranes lies slightly above the solid line obtained for untreated membranes, a linear regression analysis of the data shows that the S values before and after treatment agree fairly well (within experimental error). Plotting the maximum hyperfine splittings $2T_\parallel$ instead of the order parameter as a function of $1/T$ also gave a linear relationship with a single slope (data not shown).

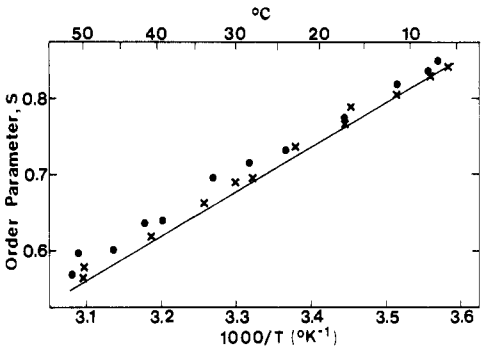

FIGURE 3: Order parameter S as a function of $1/T$ (K^{-1}) for 5-doxylstearic acid incorporated into brush border vesicle membranes. For the sake of clarity, the data points have not been included; instead, the solid line is shown that is obtained by a linear regression analysis of the data points: $y = (-1.23 \pm 0.04) + (0.58 \pm 0.01)x$ with $r^2 = 0.99$ (slope and intercept represent the mean ± standard deviation). The order parameter S of brush border membrane after papain digestion is given by (×) and that after incubation of brush border vesicles a pH 8.8 and 4 °C for 1 h by (●). The linear regression analysis of the data gives y (×) = $(-1.30 (\pm) 0.06) + (0.60 \pm 0.02)x$ with $r^2 = 0.99$ and y (●) = $(-1.14 \pm 0.07) + (0.56 \pm 0.02)x$ with $r^2 = 0.99$.

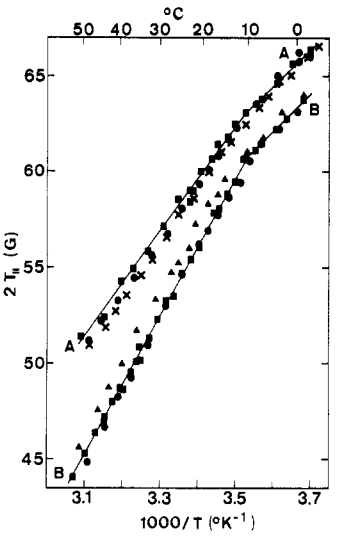

FIGURE 4: Maximum hyperfine splitting $2T_\parallel$ (G) as a function of $1/T$ (K^{-1}). Brush border vesicle membranes were labeled either with 5-doxyl-PC (top set of data points, A) or with 8-doxyl-PC (bottom set of data points, B) as described under Materials and Methods. Brush border vesicles untreated (■); brush border vesicles after papain treatment (×); brush border vesicles incubated at pH 8.8 (●); brush border vesicles after removal of actin by exposing the membrane to pH 11 (▲). Both sets of data points (A and B) are best analyzed by two linear regression lines. For clarity, only the linear regression lines for untreated brush border vesicles are shown. The two straight lines of each plot (solid lines A and B) intersect at about 10 °C (see Table I). The experimental data shown for various treatments of brush border vesicles were all analyzed by two linear regression lines, and the temperatures of the break point in the slopes thus calculated are summarized in Table I.

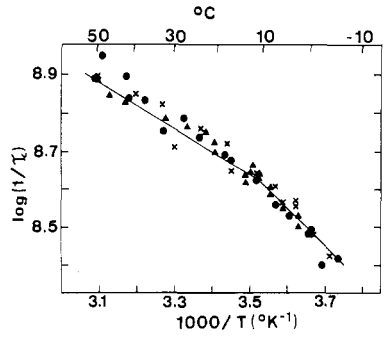

FIGURE 5: Arrhenius plot of rotational correlation time τ_c for 16-doxylstearic acid incorporated into brush border vesicle membranes. The data are best fitted by two straight lines intersecting at 11 °C (Table I). For the sake of clarity, the data points for untreated brush border vesicles have been omitted, but instead, the two linear regression lines fitted to these data points are presented (solid lines). Brush border vesicles after treatment with papain (×); brush border vesicles after incubation at pH 8.8 (●) and pH 11 (▲). Each set of data points was analyzed as described for untreated membranes, and the temperatures of the intercept of the two linear regression lines are given in Table I.

Similar results were obtained when 5-doxyl-PC was incorporated in brush border vesicle membranes. By plotting $2T_{\parallel}$ as a function of $1/T$ (Figure 4, top set of data points, A), it can be seen that the $2T_{\parallel}$ values measured in untreated membranes of brush border vesicles (■) are within experimental error, consistent with those measured after papain treatment (×) or incubation at pH 8.8 (●). Furthermore, plotting $2T_{\parallel}$ instead of S allows the temperature range to be extended to lower temperatures. In this case, statistical analysis of the data shows that they are best fitted by two straight lines intersecting at about 10 °C. Similar results were obtained when 8-doxyl-PC was incorporated into brush border vesicle membranes (Figure 4, bottom set of data, B). The $2T_{\parallel}$ values measured for brush border vesicles (■) agree within experimental error with the $2T_{\parallel}$ values of brush border vesicles incubated at pH 8.8 and 37 °C for 1 h (●). Further, the $2T_{\parallel}$ values for brush border vesicles after removal of actin by alkaline treatment [pH 11 (▲)] lie slightly above the regression line through the data points for untreated membranes. Similar to the set of data obtained with 5-doxyl-PC (top set, Figure 4), statistical analysis of the temperature dependence ($2T_{\parallel}$ vs. $1/T$) shows that the data are best fitted by two straight lines intersecting at 10 °C.

The ESR spectra for 16-doxylstearic acid incorporated in brush border vesicle membranes indicate that the label undergoes rapid and almost isotropic motion [data not shown; cf. Hauser et al. (1982)]. From the ESR spectra recorded over the temperature range 0–50 °C, orientational correlation times τ_c were calculated as described before (Hauser et al., 1982). In Figure 5, log $(1/\tau_c)$ is plotted as a function of $1/T$, for untreated brush border vesicles (—), for brush border vesicles treated with papain (×), for brush border vesicles incubated at pH 8.8 and 37 °C for 1 h (●), and for brush border vesicle membranes after removal of actin (▲). The data obtained for brush border membranes before and after the various treatments agree within experimental error. All plots appear to consist of two straight line sections with a discontinuity (Table I).

Differential Scanning Calorimetry. The thermal behavior of rabbit small intestinal brush border membrane and of the total lipids extracted from this membrane is shown in Figure 6. Brush border membranes gave reproducibly the following pattern: the first heating curve consists of a broad endothermic transition between 10 and 30 °C with a peak temperature of about 25 °C and several irreversible endothermic transitions in the temperature range of 50–80 °C. These high temperature transitions are absent in the heating curves of the lipid extract. On the basis of this and their irreversible nature, these transitions are assigned to the denaturation of membrane proteins and possibly to specific lipid–protein interactions. Upon sample cooling, a broad reversible exothermic transition is observed between 25 and 12 °C with a peak temperature

Table I: Thermal Behavior of Rabbit Small Intestinal Brush Border Membranes[a]

temperature-dependent parameter	brush border membrane	temperature of discontinuity (°C)	method
$T_{\parallel}$	untreated	10	ESR using 5-doxyl-PC
	papain treated	10	
	pH 8.8	10	
$T_{\parallel}$	untreated	10	ESR using 8-doxyl-PC
	after removal of actin	14	
	pH 8.8	11	
$1/\tau_c$	untreated	11	ESR using 16-doxylstearic acid
	papain treated	10	
	after removal of actin	12	
	pH 8.8	13	
$T_{\perp}$	untreated	0–20	ESR using cholestane spin-label
D-glucose uptake	untreated	17	activity determination
sucrase activity	untreated	no break	activity determination
phase behavior	untreated	25 (10–30)	DSC

[a] Temperatures at which discontinuities were observed in the temperature dependence of various ESR parameters and enzymatic activities are compared with the thermotropic phase transition determined by DSC. The temperature dependence of various ESR parameters was monitored over a temperature range of at least 0–50 °C. Brush border vesicle membranes were used untreated or after various treatments carried out as described under Materials and Methods. Spin-labels were incorporated into brush border membranes as described under Materials and Methods, and the spin-labeled membrane was then subjected to various treatments except for the fatty acid spin-label, which was incorporated afterward. No difference was observed in the results depending on whether the label was incorporated before or after the treatment. The treatment at pH 8.8 involves the incubation of brush border vesicles (prepared as described under Materials and Methods) at pH 8.8 and 37 °C for 60 min.

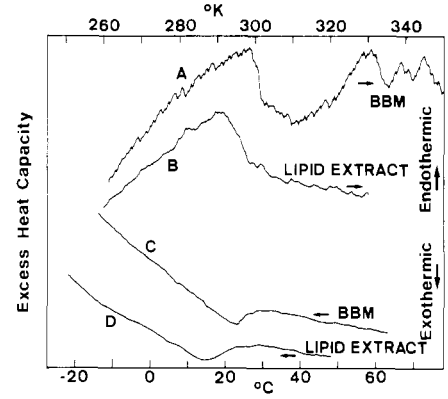

FIGURE 6: DSC thermograms of brush border vesicles dispersed in buffer as described under Materials and Methods. Heating curves recorded at 2.5 °C/min are shown for brush border vesicles (BMM, A, first heating run) and for the lipid extract dispersed in the same buffer as the membranes (B). Cooling curves recorded at 5 °C/min are shown for brush border vesicles (BBM, C) and for the lipid extract (D).

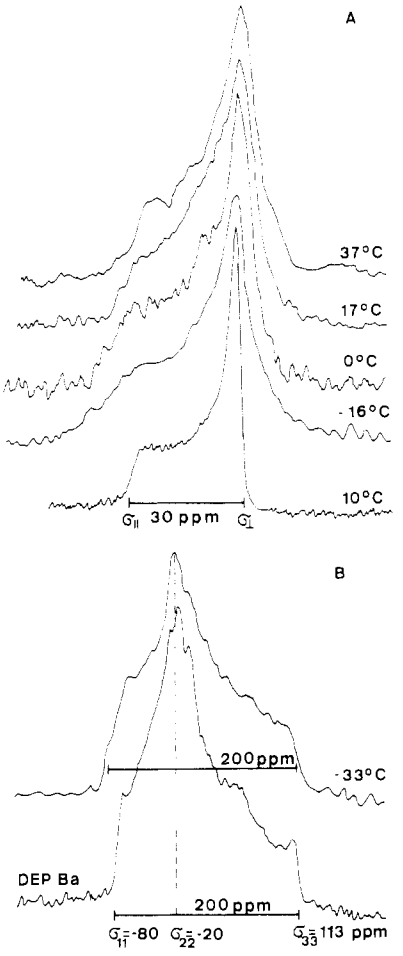

FIGURE 7: Proton-decoupled ^{31}P NMR spectra recorded at 121 MHz on a Bruker CXP 300 Fourier-transform spectrometer. Chemical shifts are measured relative to 85% orthophosphoric acid. (A) The lipids (50–100 mg) extracted from brush border vesicles were dispersed in 1 mL of buffer (10 mM Hepes/Tris, pH 7, 300 mM D-mannitol, 5 mM EDTA, 0.02% NaN_3). The sample was filled into a 5-mm NMR tube (volume ~0.2 mL), and approximately 1200 free induction decays were averaged at the temperature indicated. For comparison, the spectrum from a 10%, unsonicated 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine dispersion in H_2O is included. The shift anisotropy of this compound is $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp} \simeq -30$ ppm obtained from the computer simulation of the axially symmetric powder spectrum. (B) Axially asymmetric powder spectrum obtained from the lipid extract of brush border membranes dispersed in buffer (top). An almost superimposable spectrum was obtained from a pellet of brush border vesicles centrifuged at 60000*g* for 30 min (data not shown). For comparison, the axially asymmetric ^{31}P powder spectrum obtained from barium diethyl phosphate (DEPBa) at room temperature is included (bottom). Crystals of DEPBA were obtained as described by Herzfeld et al. (1978). The principal values of the tensor components σ_{11}, σ_{22}, and σ_{33} of DEPBa agreed within 1 ppm with the values published by Herzfeld et al. (1978) and within 3 ppm with the tensor components derived from the dispersion of the lipid extract.

of about 20 °C (curve C). Subsequent heating runs exhibit reproducible broad endothermic transitions between 17 and 33 °C with a peak temperature of about 25 °C. As mentioned above, no high-temperature transitions are observed on subsequent heating runs (data not shown). The broad transition at about 25 °C can be attributed to membrane lipids since lipids extracted from brush border vesicles and dispersed in the same buffer give similar broad reversible transitions (curve B). The lipid extract dispersed in the same buffer as brush border membrane exhibits a broad endotherm between about 2 and 30 °C with a peak temperature of about 20 °C and no high-temperature transitions. Upon sample cooling, a broad exothermic transition centered at about 14 °C is observed reproducibly (curve D of Figure 6). The enthalpy associated

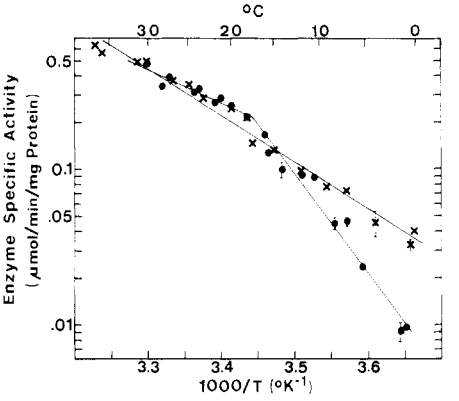

FIGURE 8: Arrhenius plots of D-glucose uptake into brush border vesicles in the presence of a Na^+ gradient (●) and of sucrase activity (×). The latter was assayed in a dispersion of brush border vesicles in buffer as described under Materials and Methods. The bars or the size of the symbols represents the spread of three experiments. The data points for D-glucose uptake are best fitted by two straight lines ($y = 8.5 - 2.2x$, $r^2 = 0.90$ for temperatures above 17 °C; $y = 22 - 6.3x$, $r^2 = 0.97$ for temperatures below 17 °C) with a discontinuity at 17 °C. The data points for sucrase activity of brush border vesicles are best fitted by a single straight line: $y = 9.6 - 3.0x$, $r^2 = 0.99$. The apparent energy of activation ΔE is obtained from the slope $b = -\Delta E/(2.3R)$ where R is the gas constant. Values for ΔE thus derived are summarized in Table II.

with the lipid transition is very small, regardless of whether it was measured in the brush border membrane or in aqueous dispersions of the lipid extract. The enthalpy values are of the same order of magnitude as those reported for brush border membranes isolated from rat small intestines (Brasitus et al., 1980). The thermal behavior of brush border membranes after papain treatment or alkaline treatment at high pH to remove actin was practically identical with that of untreated membranes (data not shown). Therefore, loss of peripheral protein amounting to ~70% of the total protein has no effect on the thermal behavior of this membrane.

^{31}P NMR. ^{31}P NMR spectra from the total lipids extracted from brush border membrane vesicles and dispersed in aqueous buffer are shown in Figure 7A. The spectra recorded over the temperature range of −16 to 37 °C are typical for a bilayer structure with the lipid molecules undergoing rapid motional averaging about the bilayer normal. Both the line width and the chemical shift anisotropy $\Delta\sigma$ increase with decreasing temperature. Temperatures well below −20 °C were required to completely freeze the motional averaging and to produce ^{31}P powder spectra typical for an axially asymmetric ^{31}P shift tensor (Figure 7B). DSC experiments using the same dispersions showed that water associated with the lipid froze at temperatures lower than −20 °C. This would suggest that the termination of the motional averaging of the lipid phosphate group coincides with the freezing of membrane-bound water. The ^{31}P NMR spectra recorded from brush border vesicle membranes are essentially similar to those shown in Figure 7 except that there is an isotropic component superimposed on the bilayer spectrum (data not shown). At least part of this isotropic component is due to lipid and protein degradation, leading to the release of small phosphate-containing compounds into the bulk solution.

Enzymatic Activities of Brush Border Membrane. The question arises as to whether the function of integral membrane proteins is affected by the lipid phase transition. To answer this question, the temperature dependence of D-glucose transport, as an example of an intrinsic membrane protein, was measured over the temperature range 0–30 °C. This measurement yielded an Arrhenius plot (Figure 8) consisting

Table II: Temperature Dependence of Enzymatic Activities in Rabbit Small Intestinal Brush Border Membrane

enzyme	activity	Arrhenius plot: apparent break point	Arrhenius plot: activation energy, ΔE (kJ/mol)
sucrase/isomaltase	sucrase	none	58 (14)[a]
D-glucose transport protein	D-glucose transport	17 °C	41.5 (10) (above 17 °C) 121 (29) (below 17 °C)

[a] The values in parentheses are in kcal/mol.

of two linear portions and a break point at 17 °C, which is within the temperature range of the thermotropic lipid phase transition (Table I). This indicates that this class of membrane proteins, which is embedded in the lipid bilayer, senses the lipid fluidity and that their enzymatic activity depends on the lipid fluidity. The apparent activation energy is 121 kJ/mol (29 kcal/mol) below 17 °C and 42 kJ/mol (10 kcal/mol) above this temperature (Table II). This behavior is contrasted by the temperature dependence of sucrase activity, which was monitored over the temperature range of 0–37 °C. The temperature dependence of this protein gives a linear Arrhenius plot with a single slope (Figure 8) and an apparent energy of activation ΔE of 58 kJ/mol (14 kcal/mol) (Table II). The value is in good agreement with the value determined in rat intestinal brush border membrane (Brasitus et al., 1979; Brasitus & Schachter, 1980). The function of this protein is apparently not affected by the lipid phase transition.

Discussion

The ESR spectra of 5-doxylstearic acid, 5-doxyl-PC, 8-doxyl-PC, and cholestane spin-label incorporated in both brush border vesicle membranes and aqueous dispersions made from their extracted lipids are similar and characteristic of rapid anisotropic motion. The ESR results are consistent with the long axis of the spin-labeled molecule being aligned essentially along the bilayer normal and the molecule undergoing rapid rotation about this axis (Hubbel & McConnell, 1969, 1971). ^{31}P NMR spectra shown in Figure 7A provide clear evidence for this. Temperatures well below −20 °C were required to completely freeze the motional averaging and to produce axially asymmetric ^{31}P powder spectra (Figure 7B). In contrast, the temperature dependence of the ESR spectra of the cholestane spin-label in brush border membranes and lipid bilayers made from the lipid extract indicates that the reorientation of the steroid nucleus about its long axis is frozen at temperatures lower than about 0 °C. Although at first sight this seems inconsistent with the ^{31}P NMR data, the difference between the ESR and NMR data could be due to the different time scale of the two techniques. Alternatively, the averaging of the ^{31}P chemical shift tensor is not due to reorientation of the phospholipid molecules as a whole but is probably due to rotation of the polar head group about one of the C–C bonds of the polar group. For example, the first glycerol bond C–CH_2OP has been postulated to be aligned almost perpendicular to the bilayer plane (Hauser et al., 1981). Rotation about this bond could account for the motional averaging observed in the ^{31}P NMR spectrum without changing the overall orientation of the head group relative to the bilayer plane (Hauser, 1981) even at temperatures at which the bulk phospholipid part of the membrane is frozen. In summary, ^{31}P NMR clearly shows that the preferred structural arrangement of brush border membrane phospholipids in aqueous dispersions is the bilayer; furthermore, most, if not all, of the phospholipid in brush border membranes occurs in this structure.

Thermal Behavior of Brush Border Membranes. The temperature dependence of various ESR spectral parameters, e.g., hyperfine splitting and the rotational correlation time, shows discontinuities consistent with results reported before (Hauser et al., 1982). Discontinuities were detected in both spin-labeled brush border membranes and spin-labeled liposomes made from the lipid extract. The fact that in both membranes the discontinuities occurred at the same or similar temperatures indicates a lipid property that is not greatly affected by the presence of membrane protein. Furthermore, the discontinuities are not only observed in the temperature dependence of different ESR parameters but also with various spin-labels greatly differing in chemical structure. On the basis of these observations, the discontinuities were previously tentatively assigned to thermotropic lipid phase transitions and/or phase separations (Hauser et al., 1982). The DSC analysis of brush border membranes and of their lipid extract supports this conclusion. In both cases, reversible broad phase transitions are observed in the range 10–30 °C, with a peak temperature of 25 °C. These transitions are characterized by a very low enthalphy, which is of the order of 1/100 of that measured for dipalmitoylphosphatidylcholine (Chapman et al., 1967; Phillips et al., 1969; Hinz & Sturtevant, 1972). Similar broad transitions of low enthalpy have been reported for rat brush border membranes (Brasitus et al., 1980), except that the peak temperature was somewhat higher (31 °C, range 23–39 °C). Ohyashiki et al. (1982) monitored the temperature dependence of –SH group accessibility, the fluorescence intensity, and the polarization of various fluorescent labels in porcine intestinal brush border membranes. Discontinuities were observed in the temperature dependence of these parameters between 30 and 35 °C, and the authors interpreted these discontinuities in terms of a lipid order–disorder transition coupled with concomitant structural changes in the membrane protein. Again, the transition temperature in porcine brush border membrane appears to be higher than that of rabbit.

The broad and low enthalpy phase transition typical for brush border membrane probably reflects a low lipid cooperativity (Oldfield & Chapman, 1972; Hinz & Sturtevant, 1972; McElhaney et al., 1973; Lee, 1975). It has been observed with other membranes, e.g., *Mycoplasma* membrane (Steim et al., 1969), bacterial membranes (Haest et al., 1974; Melchior & Steim, 1976), as well as with complex lipid mixtures. Lipid–protein interactions cannot be the main reason for the low enthalpy and the lack of cooperativity since the protein-free membrane lipids also give low enthalpy transitions. This points to the lipid composition being responsible for it. Brush border membranes isolated from rat or mouse small intestine were shown to contain a significant amount of cholesterol, amounting to 14 and 16% of the total lipid content (Forstner et al., 1968; Kawai et al., 1974; Brasitus et al., 1980). The cholesterol content of rabbit small intestinal brush border membrane was reported to be about 10% (Hauser et al., 1980) and that of chick intestinal brush border membrane 24% of the total lipid content (Max et al., 1978). Chapman and his co-workers (Ladbrooke et al., 1968; Ladbrooke & Chapman, 1969) showed that the effect of increasing quantities of cholesterol in dipalmitoylphosphatidylcholine–cholesterol mixtures is to reduce the enthalpy of the phase transition of the phospholipid; at a phospholipid to cholesterol molar ratio of 2:1, the phase transition is almost completely abolished. In a strain of *Mycoplasma mycoides* containing little cholesterol (3% of

the total membrane lipid), a lipid phase transition was observed, but no such transition occurred in the native strain that contained 22–25% cholesterol (Rottem et al., 1973). Similarly, human erythrocyte membranes, which normally contain 24% cholesterol, were shown to undergo a lipid phase transition only after removal of cholesterol (Ladbrooke et al., 1968). This consideration suggests that the cholesterol content together with the lipid heterogenity of brush border membrane are probably the main factors accounting for the low enthalpy of the lipid transition. A cholesterol content of about 10–15% is apparently sufficient to lead to a significant reduction in the enthalpy of the phase transition but not to suppress it completely. The thermal behavior of brush border membranes is summarized in Table I. Except for the cholestane spin-label, the discontinuities observed with various spin-labels correspond to the lower limit, or the onset, of the transition temperature range determined by DSC. Similar observations were made in rat brush border membrane (Brasitus et al., 1980). The discontinuities determined with different spin probes and different spectral parameters are in good agreement with each other (Table I; average = 11 ± 1.5 °C). Previously, when different fatty acid spin probes with the doxyl group at a different depth along the bilayer normal were used, a large spread (10–30 °C) in the temperatures was observed at which discontinuities occurred (Hauser et al., 1982).

The temperature dependence of the maximum hyperfine splitting of the cholestane spin-label gives a temperature range for the transition rather than a single discontinuity. In the transition range 0 to about 20 °C (Table I), the experimental spectra are composite, consisting of at least two component spectra. One possible explanation is that the phase transition is accompanied by a lateral phase separation. As a result, the label would report two or more environments provided the exchange rate between the different environments is slow on the ESR time scale.

It is clear from Table I that various treatments of brush border vesicles leading to substantial losses of membrane-bound enzymes and other proteins have little if any effect on the thermotropic lipid phase transition. Papain treatment of brush border vesicles as used here has been shown to release essentially all sucrase/isomaltase activity and also other hydrolase activity in water-soluble form (Sememza et al., 1977; Tannenbaum et al., 1977). This amounts to a protein loss of about 65%. There is evidence that papain cleaves sucrase/isomaltase near the NH_2-terminal end of the isomaltase. The relatively small hydrophobic NH_2-terminal peptide (probably less than 10% of the total sucrase/isomaltase) is unaffected by the papain treatment and remains associated with the lipid bilayer while the bulk of the protein together with the two active centers are released as a water-soluble, globular protein (Brunner et al., 1979). If this mechanism of papain cleavage is a general one, then it is expected that the removal of the extrinsic portion of these membrane-bound proteins has no effect on the fluidity and packing of brush border membrane. Incubation of brush border membrane at alkaline pH also leads to the release of a sizable proportion of the total vesicle protein. Exposure of brush border vesicles to pH 8.8 and 11 had very little effect on the membrane fluidity and packing; furthermore, the thermal behavior of the lipid part of the membrane was practically unaffected (Table I; cf. Figures 3–5). This result suggests that the alkaline treatment releases essentially peripheral (extrinsic) proteins bound to the membrane lipid or integral membrane protein by electrostatic forces and not by hydrophobic interaction. Breaking of the links between these peripheral proteins and integral constituents of the membrane apparently does not affect the membrane fluidity and packing.

Figure 8 sheds light on the question of whether or not the function of integral membrane proteins is affected by the lipid phase transition. While the Arrhenius plot of the D-glucose transport system shows a discontinuity, the sucrase activity of the membrane-bound enzyme sucrase/isomaltase is apparently not affected by the lipid phase transition. The sucrase activity and its temperature dependence were shown to be the same (within experimental error) in the papain-solubilized and in the membrane-bound enzyme (Semenza, 1976). This is not surprising considering the model of the insertion of sucrase/isomaltase in the lipid bilayer discussed above (Brunner et al., 1978, 1979). Studying the temperature dependence of various enzymes in rat intestinal brush border membranes, Brasitus et al. (1979; Brasitus & Schachter, 1980) also showed that these enzymes can be grouped into two classes according to their Arrhenius plots: enzymes giving Arrhenius plots with a single slope and enzymes giving Arrhenius plots with a break point. These authors proposed to use this criterion to differentiate between extrinsic and intrinsic membrane enzymes, respectively.

The ESR spin-label results and the ^{31}P NMR measurements shed light on the molecular mechanism that underlies the thermotropic lipid phase transition. The temperature dependence of the ESR spectra from cholestane clearly shows that at temperatures below the transition region (hatched area, Figure 2), the rotational motion about the long axis of the steroid nucleus is frozen. It is reasonable to assume that the motion of the steroid spin probe is representative of the average motion of the lipid molecules in the membrane (Seelig, 1976). If this is correct, the ESR results can be interpreted to indicate that cooling below the transition temperature (~25 °C) induces a transition from the liquid-crystalline to the gel state with crystalline hydrocarbon chain packing. The ESR results with the cholestane spin-label suggest, but do not prove, that the transition is accompanied by lateral phase separation with lipid cluster formation. The temperature dependence of the ^{31}P NMR spectra indicate that there is still segmental motion in the phospholipid polar group in the gel phase well below 0 °C. In conclusion, there are three lines of evidence that support a lipid thermotropic phase transition: differential scanning calorimetry, the temperature dependence of ESR spectral parameters, and the temperature dependence of the activities of integral membrane proteins. The lipids of the rabbit small intestinal brush border membrane undergo a broad, reversible phase transition of low enthalpy between 10 and 30 °C with a peak temperature of about 25 °C. This indicates that the brush border membrane functions at a temperature well above the lipid phase transition.

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