Electron Spin Resonance Evidence for Vertical Asymmetry in Animal Cell Membranes†

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ABSTRACT: Two electron spin resonance (ESR) spin labels were used to monitor the physical state of bacterial and animal cell membranes: 5N10, a nitroxide derivative of decane, and 12NS-GA, a glucosamine derivative of 12-nitroxide stearic acid. Spectra were recorded at 1 °C intervals from ~5 to 45 °C. Arrhenius plots of log h_H/h_P vs. $1/K$ were obtained by measuring the amplitudes of the hydrocarbon and water signals, h_H and h_P, respectively. Two discontinuities in the Arrhenius plot (at characteristic temperatures t_l and t_h) were observed with bacterial cell membranes independent of the spin label employed. Analysis of sealed animal cell membrane samples revealed four characteristic temperatures when the hydrophobic spin label 5N10 was used, but only two when the amphiphilic spin label 12NS-GA was used. The specific set of characteristic temperatures revealed with 12NS-GA depended on whether the membrane preparation was inside out (ISO) or right side out (RSO). Analysis of Newcastle disease virus, a source of RSO plasma membrane derived from host, revealed two characteristic temperatures at ~14 and 33 °C. Analysis of phagosomes, a source of ISO plasma membrane derived from LM cells, revealed two characteristic temperatures at ~23 and 38 °C. When unsealed or disrupted membrane preparations were spin labeled with 12NS-GA, both sets (RSO and ISO) of characteristic temperatures were revealed. The results indicate that the inner and outer monolayers of animal cell membranes are physically distinct and that the glycosylated spin label, 12NS-GA, is apparently restricted in its ability to flip across the membrane bilayer. In this study, characteristic temperatures were pinpointed by computer analysis of the ESR spectral data.

The usefulness of electron spin resonance spectroscopy (ESR)[1] in analyzing the physical properties of membrane lipids stems from the nitroxide spin-labeling experiments of McConnell and co-workers (Hubbell and McConnell, 1968; Shimshick and McConnell, 1973). These investigators established the partitioning of certain nitroxide spin labels between hydrocarbon and aqueous domains of model membrane systems. Arrhenius plots of the relative fraction of spin label in hydrocarbon as a function of temperature showed discontinuities at characteristic temperatures. These temperatures, t_l and t_h, defined the onset and completion of the lateral phase separation process of lipids in binary systems as well as in *E. coli* membrane preparations (Linden et al., 1973b). Phase diagrams constructed for the *E. coli* system have since been verified by x-ray diffraction (Linden and Fox, 1975).

In an attempt to define the physical characteristic temperatures of animal cell membranes, Wisnieski et al. (1974b) used the spin-labeling method of Linden et al. (1973a) in which temperature-dependent partitioning of the spin label 5N10 (a nitroxide derivative of decane) was monitored by ESR. The animal cell membrane studies focused on plasma membranes and endoplasmic reticulum membranes from mouse fibroblasts (LM cells), and Newcastle disease virus (NDV) harvested from embryonated chick eggs. NDV, a membrane-enveloped paramyxovirus whose membrane lipid is derived from plasma membrane of host cells (Choppin et al., 1971; Blough and Lawson, 1968), represented a source of chick plasma membrane. Critical analysis of ESR data of multiple samples from each membrane type revealed the existence of four characteristic temperatures in each of the three membrane preparations (Wisnieski et al., 1974a,b). The characteristic temperatures for LM plasma membrane occurred at ~15, 21, 30, and 37 °C. Those for LM endoplasmic reticulum were 1–2 °C higher as were those for chick plasma membrane (envelope of NDV). To account for the four characteristic temperatures, a membrane physical asymmetry model was proposed. The model was supported by physiological data on cis and trans plasma membrane activities and by a mathematical assessment of ESR spectral data (Wisnieski et al., 1974a,b; Fox, 1975). According to the model, the t_l and t_h of one monolayer occur at approximately 15 and 30 °C, and the t_l and t_h of the other monolayer occur at approximately 21 and 37 °C.

The asymmetry model proposed for animal cell membranes has a precedent in studies showing asymmetry of phospholipid head group distribution in human erythrocyte ghosts (Bretscher, 1972, 1973; Verkleij et al., 1973; Rothman and Dawidowicz, 1975) and in several membrane-enveloped viruses (Tsai and Lenard, 1975; Lenard and Rothman, 1976). For example, Bretscher (1972, 1973) and van Deenen and his associates (Verkleij et al., 1973) have made observations consistent with asymmetric distribution of phospholipids across the membrane bilayer of the human erythrocyte. Phospholipase C treatment and phospholipid exchange are two common methods employed in studies of this type.

Since the concept of vertical membrane physical asymmetry is a novel idea in membrane biology, and because recent evi-

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[1] Abbreviations used: ESR, electron spin resonance spectroscopy; 5N10, 2-butyl-2-pentyl-4,4-dimethyl-3-oxazolidinyloxy; 12NS, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy; 12NS-GA, glucosamine derivative of 12NS; DDTAB, 4-(dodecyldimethylammonium)-2,2,6,6-tetramethylpiperidinyl-1-oxy bromide; NDV, Newcastle disease virus; TLC, thin-layer chromatography; ISO, inside out; RSO, right-side out; DPPC, dipalmitoylphosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane.

dence suggests that many important properties of animal cell membranes may depend on lateral diffusion and phase separation of membrane components, we designed an experimental approach to define the precise characteristic temperatures at which a change in physical state occurs in each monolayer. This approach exploited right-side out (RSO) and inside out (ISO) membrane preparations and a spin label attached to a large polar group to restrict its ability to flip from an outer to an inner monolayer (Iwata et al., 1976).

Materials and Methods

ESR Spin Labels. The hydrocarbon spin label 5N10 (2-butyl-2-pentyl-4,4-dimethyl-3-oxazolidinyloxy) was a gift from Dr. A. D. Keith, Pennsylvania State University. The quaternary amine spin label DDTAB [4-(dodecyldimethylammonium)-2,2,6,6-tetramethylpiperdinyl-1-oxy bromide] was a gift from Dr. R. Mehlhorn, University of California, Berkeley. The fatty acid spin label 12NS [2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy] was purchased from Synvar, Palo Alto, Calif.

The glucosamine (GA) derivative of 12NS (i.e., 12NS-GA) was prepared by two different methods. The first method involved adding 13.2 mg of dicyclohexylcarbodiimide (Aldrich) in 66 μL of pyridine to 25 mg of 12NS. Next, 685 μL of a 1 M stock of GA (Sigma) in Me_2SO was added and the reaction tube vortexed for 5 min. Pyridine was evaporated off at room temperature and 1 mL of $CHCl_3$–MeOH (2:1) was added. The sample was then filtered through Na_2SO_4–glass wool using $CHCl_3$–MeOH (2:1) as a wash. The filtrate was evaporated and the residue picked up in a small amount of $CHCl_3$–MeOH. This was applied to a chromatography column containing 5 g of MN-Kieselgel (70–325 mesh ASTM) suspended in ether. The column was washed with 40 mL of ether and then with 40 mL of $CHCl_3$–MeOH (2:1). The latter fraction was evaporated and applied to a thin-layer chromatography plate (silica gel G, 500 μm) which was developed in $CHCl_3$–MeOH–water (65:35:5). The 12NS-GA band (R_f approximately 0.82) was scraped from the plate, suspended in $CHCl_3$–MeOH (2:1), and filtered through Na_2SO_4–glass wool. The filtrate was dried down under N_2 and suspended in ethanol.

The second method of synthesizing 12NS-GA involved adding glucosamine hydrochloride (14 mg in 0.156 mL of water or in 0.056 mL of Me_2SO) to a solution containing 80 mg of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, Calbiochem) and 25 mg of 12NS in 0.34 mL of absolute ethanol. The sealed reaction mixture was shaken (200 rpm) first at 37 °C for 2 h and then at room temperature overnight. An excess of benzene was added and the solvents were evaporated at room temperature. The residue was washed with approximately 10 mL of ether and filtered through a Whatman No. 40 ashless filter paper. The ether insoluble material was washed with acetone and fractions which gave a positive test for *N*-acylamino sugars (see below) were pooled and dried under N_2. 12NS-GA was then purified by thin-layer chromatography as described in the first method.

Preliminary identification of 12NS-GA was performed by analyzing the molar ratio of ^{3}H-labeled fatty acid:[^{14}C]glucosamine in each product of a radioactive mixture separated by two-dimensional thin-layer chromatography using ether vs. $CHCl_3$–MeOH–water (65:35:5). The product identified as 12NS-GA was resistant to mild alkaline hydrolysis and satisfied a colorimetric assay for *N*-acylamino sugars (Reissig et al., 1955). The structure and molecular weight were then verified by mass spectroscopy of the permethylated derivative. Samples were permethylated as follows: 0.1 g of ether-washed NaH in 1 mL of Me_2SO was heated at 80 °C for 20 min and centrifuged, and the supernatant was removed and stored under N_2. Sample (200 μg; 12NS-GA) was treated with 50 μL of the above solution at 25 °C for 30 min. CH_3I or iodobenzene (50 μL) was added and this reaction mixture held at 25 °C for 30 min. Next, 100 μL of water was added, followed by 100 μL of $CHCl_3$. After mixing, the water phase was removed and subjected to three more $CHCl_3$ washes. The $CHCl_3$ phases were pooled, subjected to five water washes, dried, and stored under N_2 until analysis on an AEI mass spectrometer.

ESR Samples. Lipid dispersions consisted of 300 μg of dipalmitoylphosphatidylcholine in 80 μL of a Tris–saline solution (Wisnieski et al., 1974a) containing 0.25 mM 12NS-GA. Dispersal was achieved by mild sonication.

E. coli inner membranes (band I) were prepared from strain 30E βox$^-$ and were rich in phospholipids containing oleate (Δ^9-*cis*-octadecenoic acid). The membrane preparation was donated by Dr. C. F. Linden and was similar to that described in an earlier study (Linden et al., 1973a).

Newcastle disease virus (NDV), strain HP-16, was propagated in embryonated chick eggs and purified according to Samson and Fox (1973) with an additional purification on a renograffin gradient 20% lighter than the previous gradient. Polyacrylamide gel electrophoresis of sodium dodecyl sulfate solubilized virus revealed only viral protein bands.

Mouse LM cells were grown in suspension culture in Eagle's minimal essential medium plus 0.5% peptone. Latex beads (Dow; diameter 0.721 μm) were mixed with cells. Beads with attached plasma membrane fragments, or phagocytized beads (phagosomes) with ISO plasma membranes, were obtained by manipulating incubation time and temperature (Huang et al., 1976). Since beads are not internalized at 20 °C, plasma membrane fragments attached to beads were obtained from cell–bead mixtures incubated at 20 °C for 120 min. Beads with ISO membranes, relatively free of lysosomal enzymes, were obtained from a cell–bead mixture incubated at 30 °C for 20 min. The latex bead samples were generously supplied by Dr. Y. O. Huang. The preparation and enzymic characterization of phagosomes from LM cells will be described elsewhere (Huang, Hoff, Wisnieski, and Fox, manuscript in preparation).

All spin-label stocks were 10 mM in ethanol. Aliquots of spin label (0.5–2 μL) were added to acid-washed capillary tubes and dried down under N_2. The volume of the aliquot was based on a final sample concentration of 0.1–0.2 mM spin label. Membrane samples (50–100 μL) containing 50–200 μg of protein were added to the capillary tubes and loosely pelleted by centrifugation (Wisnieski et al., 1974a,b). All but 5–10 μL of supernatant was drawn off and discarded.

Two hundred microliters (72 μg of protein) of the 30 °C, 20-min phagosome preparation was added to 700 μL of 0.25 M sucrose solution or 700 μL of Tris–saline (pH 7.2–7.4). The sample was placed in an ice bath and sonicated for 45 s (3 s followed by 12 s of cooling, 15 times). Sonication was performed using a microtip probe attached to a Branson W185 Sonifier at setting 7 (40% output). The phagosome sample was concentrated by centrifugation. The concentrated spin-labeled sample was added to a capillary tube for spectral analysis.

ESR Spectra. ESR spectra were obtained on a Varian E104 spectrometer operating at *X* band and equipped with a Varian variable temperature accessory. Temperature was monitored (±0.1 °C) with a copper–constantan thermocouple attached to a Doric digital thermocouple indicator. Each spectrum represents a 4-min scan over a 100-G range (power = 20 mW). Multiple scans were taken at each temperature from ap-

TABLE I: A Summary of the Results of Spin-Labeling Experiments Using 5N10, 12NS-GA, and DDTAB on Various RSO, ISO, and Disrupted Membrane Preparations.

Sample[a]	Spin Label[b]	Computer-Derived Characteristic Temp (°C)[c]			
DPPC liposomes	5N10				41.0
DPPC liposomes	DDTAB				41.0
DPPC liposomes	12NS-GA				41.0
E. coli inner membrane	5N10	14.1		29.4	
E. coli inner membrane	12NS-GA	15.0		26.4	
Newcastle disease virus (RSO)	5N10	14.0	22.0	32.0	38.0
Newcastle disease virus (RSO)	12NS-GA	14.1		32.9	
Newcastle disease virus (RSO)	DDTAB	16.7	22.6	28.5	36.7
Unsonicated phagosomes (ISO)	5N10	11.3	21.6	28.8	38.7
Unsonicated phagosomes (ISO)	12NS-GA		22.6		38.3
Sonicated phagosomes	12NS-GA	12.7	22.3	29.2	38.3
LM membrane fragments attached to latex beads	12NS-GA	12.4	22.7	29.7	35.7

[a] See Materials and Methods for sample preparation. [b] Aqueous solutions of spin labels show linear Arrhenius plots of log $\tau_c = 1/K$ in the 5–45 °C range (see Results). [c] For determination of characteristic temperatures, see Materials and Methods.

proximately 4 to 45 °C. Deviations from the mean were within the circumference of the data point. ESR running time was about 6 h/sample.

Computer Analysis of ESR Spectral Data. The Arrhenius function log $y = 1/K$, where $y = h_H/h_p$ or f', was divided into multiple overlapping segments for inspection, e.g., 5–20, 15–30, 20–37, and 30–45 °C. For a given segment, the first analysis was a simultaneous test for equality of slopes and intercepts (computer program PMDPIR, Dixon, 1975). If the slopes and/or intercepts were inconsistent with a single line in a given segment ($p < 0.01$), the segment was subjected to curve splitting to obtain minimum residual sum of squares (best fitting line intervals). The latter involved a statistical treatment suggested by Dr. J. Raisons (unpublished data). Individual t-tests for equality of slopes and equality of intercepts were performed to determine if the two intervals obtained by curve splitting differed significantly ($p < 0.05$). The computer program for curve splitting is available upon request. Characteristic temperatures are computer-derived intersects or discontinuities in the Arrhenius function. Analysis of the characteristic temperatures from independent runs carried out in triplicate show agreement to within ±0.5 °C. Although every characteristic temperature in a given Arrhenius plot may not be visually dramatic, each characteristic temperature can be easily seen in at least two out of three similarly obtained plots.

Results

Spin Label–Water Systems. When the correlation times τ_c (Kivelson, 1960) of each spin label (0.1–0.3 mM) in Tris–saline or linoleic acid ($\Delta^{9,12}$-*cis,cis*-octadecadienoic acid) were measured as a function of temperature (0–45 °C), no discontinuities were observed in Arrhenius plots of log τ_c vs. $1/K$. Linearity of rotational time τ_c in these plots demonstrates that aqueous phase and hydrocarbon phase discontinuities are not an inherent feature of the various spin labels used in this study. Since spin–spin interactions were not apparent, we presume the concentration employed is one where the monomer is soluble.

Liposomes. Liposome samples consisting of DPPC in Tris–saline were spin labeled with 5N10, DDTAB, and 12NS-GA and the ESR spectra measured as a function of temperature. All three spin labels showed spectra similar to those obtained when 5N10 partitions (Linden et al., 1973a; Wisnieski et al., 1974a). The ratios of the amplitudes of the high-field hyperfine peaks, h_H and h_p, were measured as a function of temperature and graphed on Arrhenius plots (data not shown). The only discontinuity obtained was at approximately 41 °C independent of spin label (Table I). Consequently, analysis of a DPPC liposome system by monitoring 5N10, DDTAB, or 12NS-GA partitioning between hydrocarbon and aqueous domains as a function of temperature provides an accurate determination of lipid melting point.

E. coli Membranes. The first derivative ESR spectrum of 12NS-GA in DPPC vesicle preparations looks very similar to that of 5N10 in DPPC vesicle preparations. Resolution of aqueous and hydrocarbon signals is observed and signals arising from both fluid and solid hydrocarbon are relatively isotropic (see Linden et al., 1973a; Wisnieski et al., 1974a). Biological membranes spin labeled with 5N10 or DDTAB display spectra similar to those observed with liposome membrane systems.

When biological membranes are spin labeled with 12NS-GA, however, the spin label shows some degree of anisotropy and third line heights become too small to measure accurately. Because of the problem of resolving aqueous and hydrocarbon signals in the third line, a new spectral parameter f' based on low-field hyperfine splitting was used to monitor the physical behavior of 12NS-GA in biological membrane samples. Measurement of this empirical parameter is described in Figure 1 (top). To test its validity, *E. coli* inner membranes (band I; Materials and Methods) derived from fatty acid desaturase auxotrophs grown in oleate supplemented medium were examined using the spin label 5N10 and then 12NS-GA. With 5N10, h_H/h_p based on the high-field hyperfine lines was plotted as described by Wisnieski et al. (1974a) and two characteristic temperatures were observed in the Arrhenius plot. Next, a sample of membranes spin labeled with 12NS-GA was examined and $f' = h_H/h_p$ based on the low-field hyperfine lines was plotted (Figure 1, bottom). Arrhenius plots derived with 12NS-GA displayed discontinuities at characteristic temperatures similar to those derived with 5N10, namely, at approximately 14.5 and 28 °C. Table I summarizes data from these experiments.

Animal Cell Membranes. In contrast to the ESR studies with *E. coli* membranes, animal cell membrane systems such as LM cell plasma membranes and endoplasmic reticulum membranes as well as egg grown NDV, a source of chick cell

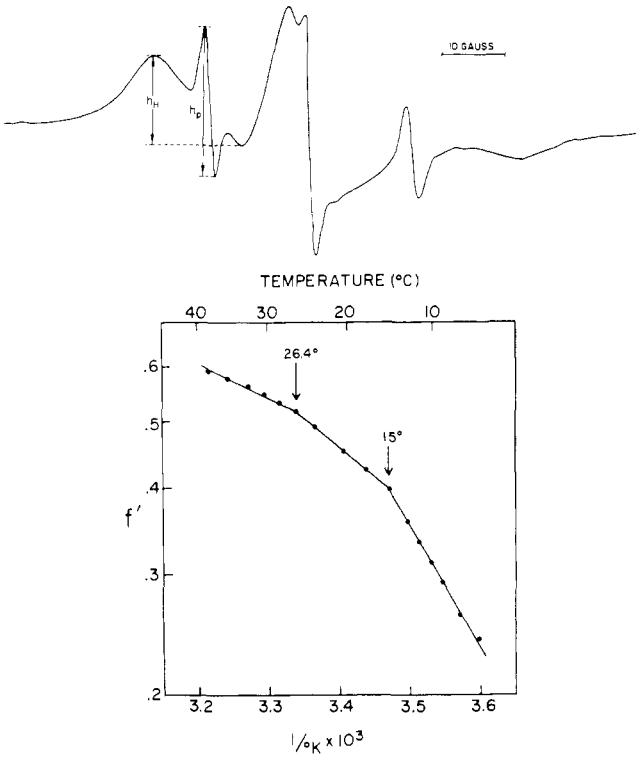

FIGURE 1: (Top) ESR spectrum at 23 °C of an aqueous suspension of NDV membranes labeled with 12NS-GA (see Materials and Methods). The empirical fluidity factor f' is defined as the ratio of the peak heights derived from hyperfine splitting of the low-field signal (i.e., $f' = h_H/h_p$). This factor reflects the relative presence of the spin label in membranous vs. aqueous domains. (Bottom) Arrhenius plot of the 12NS-GA spectral parameter f' for *E. coli* inner membranes isolated from cells supplemented with oleic acid (Materials and Methods; Linden et al., 1973a).

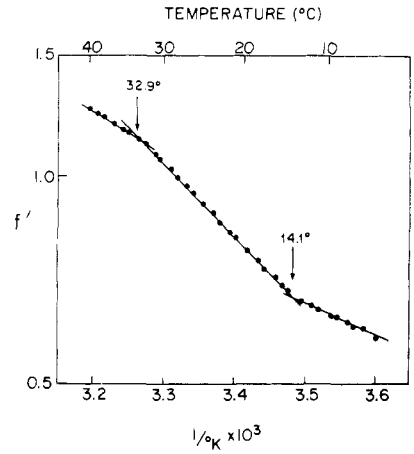

FIGURE 2: Arrhenius plot of the 12NS-GA spectral parameter f' for Newcastle disease virus.

plasma membrane, displayed at least four characteristic temperatures (~15, 21, 30, and 38 °C) by 5N10 partitioning (Wisnieski et al., 1974a,b). Table I shows the four characteristic temperatures observed with 5N10 labeled NDV. The characteristic temperatures were derived from a computer analysis of the Arrhenius plot as described in Materials and Methods. The envelope of NDV represents right side out (RSO) chick plasma membrane. In contrast to the results with 5N10, an NDV suspension spin labeled with 12NS-GA showed only two characteristic temperatures, at 14.1 and 32.9 °C ($p \ll 0.05$) (Figure 2).

With the spin-label DDTAB, a molecule purportedly restrained in its ability to cross from the outer to the inner membrane monolayer, four characteristic temperatures were observed with NDV samples. This suggests that DDTAB undergoes some "flip-flop" (Kornberg and McConnell, 1971) in the NDV system. The $t_{1/2}$ for flipping of DDTAB in egg lecithin liposomes is reportedly between 2 and 3 h at 37 °C (R. Mehlhorn, unpublished data). Table I summarizes the results of DDTAB analysis of NDV characteristic temperatures.

To examine undisrupted ISO plasma membranes, we employed LM cell plasma membranes surrounding latex beads which had been phagocytized by the cell and then isolated as phagosomes. With the spin label 5N10, four characteristic temperatures were observed (Figure 3A); with the spin label 12NS-GA, only two of these characteristic temperatures were detected (Figure 3B), at 22.6 and 38.3 °C (see Table I). However, when an identical phagosome preparation was disrupted in the presence of 12NS-GA before ESR analysis, four characteristic temperatures were observed (Figure 3C): the two seen with undisrupted ISO samples plus the two seen with 12NS-GA labeled RSO membranes (NDV). The four characteristic temperatures of disrupted phagosomes were almost identical with those revealed with 5N10 spin labeled NDV or with 5N10 spin labeled undisrupted phagosomes (Table I). A preparation of LM cell plasma membranes obtained by allowing beads to stick to cells at temperatures where phagocytosis is inhibited contained open membrane fragments and vesicles attached to beads. With either 5N10 or 12NS-GA spin labeling, these membrane fragments displayed all four characteristic temperatures of LM cell plasma membranes (Figure 3D; Table I).

As a control, "naked" latex bead suspensions (4.4×10^9 beads/50 μL of Tris-saline) were spin labeled (0.1 mM 5N10 or 12NS-GA), pelleted, and examined by ESR at 5 °C intervals from 5 to 45 °C. With 5N10, spectra contained only aqueous signals; with 12NS-GA, spectra consisted of small aqueous signals superimposed on a large spin broadened signal. Neither spin label showed any indication of solubility in latex hydrocarbon.

Discussion

There is ample evidence for vertical asymmetry of membrane lipids in the human erythrocyte ghost and in the membrane envelope of influenza A_0 virus (Tsai and Lenard, 1975; Lenard and Rothman, 1976). Inherent in the concept of compositional asymmetry is the potential for physical asymmetry. Unfortunately, little has been done by way of adapting existing technology to the problem of membrane physical asymmetry. Physical technologies offering at least the resolution to study the problem include x-ray diffraction, fluorescence spectroscopy, NMR, and ESR. The first evidence suggesting that at least some biological membranes might display physical asymmetry was based on ESR spin-labeling techniques. Data from these studies showed that animal cell membranes have at least four characteristic temperatures at which changes in physical state occur. Companion studies of several membrane-associated activities revealed thermotropic behavior consistent with the ESR observations (Wisnieski et al., 1974a,b). An analysis of available data, both physical and physiological, led to the vertical asymmetry model of animal cell membranes (Wisnieski et al., 1974a,b; Wisnieski and Fox, 1976). The present study was undertaken to test the validity of this model. It was facilitated by the development of the amphiphilic spin label 12NS-GA (Iwata et al., 1976), a car-

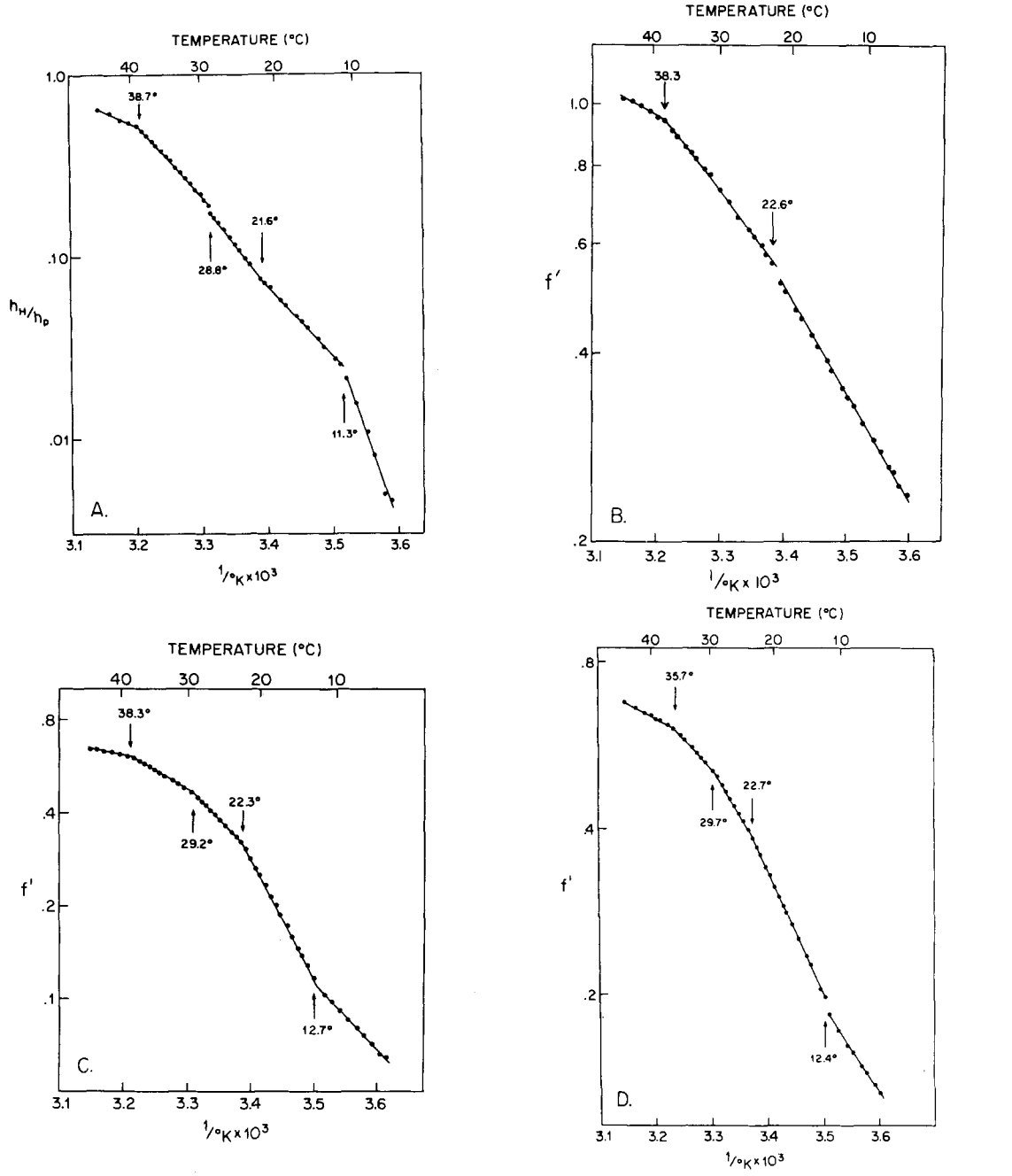

FIGURE 3: (A) Arrhenius plot of the 5N10 partitioning parameter, h_H/h_p (Wisnieski et al., 1974a), for intact phagosomes derived from LM cells. (B) Arrhenius plot of the 12NS-GA spectral parameter f' for intact phagosomes derived from LM cells. (C) Arrhenius plot of the 12NS-GA spectral parameter f' for sonicated phagosomes derived from LM cells. (D) Arrhenius plot of the 12NS-GA spectral parameter f' for LM plasma membrane fragments attached to latex beads.

bohydrate derivative of 12-nitroxide stearic acid (12NS) which, unlike 5N10, appears to be restricted in its ability to "flip" across a membrane.

The spin label 12NS-GA has a precedent in the surface restricted spin labels developed by Keith, Mehlhorn, and associates (Lepock et al., 1975; Mehlhorn and Packer, 1976). These investigators have developed quaternary amine and sulfonated derivatives of nitroxide fatty acids and alkyls. Research on ionic spin labels suggests a limited ability to "flip" but the $t_{1/2}$'s for "flipping" were not reported. Our results with the quaternary amine spin label DDTAB are not consistent with a very long $t_{1/2}$ (see NDV results).

By comparing the results of spin-labeling experiments using 5N10 and 12NS-GA on ISO, RSO, and disrupted membrane systems, we were able to define the characteristic temperatures associated with the inner and outer monolayers of animal cell plasma membranes. To facilitate an unbiased determination of characteristic temperatures, we subjected all spectral data to computer analysis. The characteristic temperatures for the outer monolayer of chick plasma membrane (NDV envelope) and for LM cell plasma membrane occur at approximately 13 and 32 °C; the characteristic temperatures for the inner monolayer occur at approximately 22 and 38 °C. The fact that each monolayer is distinguished by a unique set of characteristic temperatures indicates that animal cell membranes display vertical physical asymmetry. Thus, at all temperatures, the outer membrane monolayer is probably less rigid than the inner monolayer. This state could result from a lower pro-

tein/phospholipid ratio in the outer monolayer and/or the presence of phospholipids with lower melting points.

We have found that the spin label 12NS-GA and several analogues are not toxic to monolayer LM cells at concentrations up to 40 μg/mL (Vlodavsky and Wisnieski, unpublished data). Since *N*-stearoylglucosamine has no emulsifying properties (Fieser et al., 1956), the nitroxide derivative 12NS-GA should not have any detergent-like effects on the membrane preparations examined in the present study. Electron microscopy was performed on thin sections of samples before and after ESR analysis and no abnormalities were observed (Hoff, Iwata, and Wisnieski, unpublished data). Moreover, 12NS-GA effected no loss of viral proteins as monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Preliminary studies using 12NS-GA and mouse B lymphocytes (specifically, IgG2 bearing cells) revealed only two characteristic temperatures at approximately 14 and 28 °C. Lymphocytes spin labeled with 5N10 displayed the usual four characteristic temperatures (Krolick et al., 1977). These preliminary results suggest that 12NS-GA is a potentially valuable membrane surface probe for intact cells.

Acknowledgments

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