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Lateral Diffusion of a Hydrophobic Peptide, *N*-4-Nitrobenz-2-oxa-1,3-diazole Gramicidin S, in Phospholipid Multibilayers†

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ABSTRACT: Lateral diffusion of *N*-4-nitrobenz-2-oxa-1,3-diazole gramicidin S (NBD-GS) in phospholipid multibilayers has been studied by the method of fluorescence recovery after photobleaching. At 24 °C the diffusion coefficient (D) in egg phosphatidylcholine (EPC) multibilayers is 3.5×10^{-8} cm^2/s. Addition of equimolar cholesterol to EPC bilayers reduced D to 1.4×10^{-8} cm^2/s. In dimyristoylphosphatidylcholine (DMPC) multibilayers, the phase transition was observed as an 100-fold change in D occurring at about 22 °C. Increasing the gramicidin S (GS) membrane concentration lowered the phase transition temperature (T_m) while broadening the transition range. The transition curves constructed with the D's for NBD-GS were correlated with differential scanning calorimetry data for identical GS/DMPC mixtures. Calorimetric data for these mixtures gave multiple melting components at high GS/DMPC but when GS was mixed with dipalmitoylphosphatidylcholine (DPPC), only a single melting component was observed with a slightly decreased T_m at high GS/DPPC. However, at these high ratios, fluorescence images of NBD-GS indicated incomplete incorporation of the peptide into DPPC; this was verified by quantitating the amount of GS associated with multilamellar lipid vesicles following fractionation on an agarose column or on a sucrose gradient. In contrast, no evidence for such incomplete mixing was obtained for GS/DMPC mixtures in either multibilayers or multilamellar vesicles. Addition of cholesterol to DMPC multibilayers reduced D above T_m, raised D below T_m, and also tended to force the NBD-GS out of the bilayers. At 50 mol % cholesterol, fluorescence microscopy showed that the NBD-GS was forced to reside in the domain boundaries of the multibilayer structure. In contrast, 50 mol % cholesterol did not displace NBD-GS in EPC multibilayers as evidenced by uniform fluorescence distribution. The effect of incorporated GS on lipid mobility was studied by measuring the diffusion coefficient of NBD-phosphatidylethanolamine (NBD-PE) in EPC multibilayers at 25 °C. At an apparent concentration of 20 mol % of GS, the D of NBD-PE was reduced by a factor of 1.5 from the value obtained ($\sim 4 \times 10^{-8}$ cm^2/s) in the absence of GS. The relationship of the measured lateral diffusion to the molecular size and lipid "viscosity" is discussed.

Gramicidin S (GS)[1] is a hydrophobic, cyclic decapeptide having a molecular weight of 1141. It has been the subject of numerous structural and synthetic studies, although the mechanism of its antibiotic action is not fully understood (for review, see Kato & Izumiya, 1974). As part of our program to investigate the lateral mobility of various molecules and macromolecules in cell membranes, we have studied the diffusion of this peptide in phospholipid multibilayers by the method of fluorescence recovery after photobleaching (FRAP). This method has been described in detail in the recent literature (Jacobson et al., 1977; Koppel et al., 1976; Edidin et al., 1976). A major purpose of these studies on model membranes is to understand factors governing the lateral diffusion of phospholipids, peptides, and proteins in membranes of defined lipid composition in order to provide a base line with which to compare and interpret the lateral mobility results obtained from measurements on the membranes of single, living cells (see, for example, Edidin et al., 1976; Jacobson et al., 1976, 1977; Schlessinger et al., 1976, 1977; Axelrod et al., 1976b). Studies of lipid diffusion in multibilayers (Wu et al., 1977) and black lipid membranes (Fahey et al., 1977; Wolf et al., 1977) as well as the diffusion of stearoylated dextrans bound to black lipid membranes (Wolf et al., 1977) have already been reported. In this report, the first to our knowledge on peptide lateral mobility in lipid bilayers, we present data on the effect of a bilayer phase transition and the effect of cholesterol on the lateral diffusion of NBD-GS in lipid multibilayers. We also studied the effect of gramicidin S on the lateral diffusion of a fluorescent phospholipid analogue.

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; GS, gramicidin S; NBD-GS, *N*-4-nitrobenz-2-oxa-1,3-diazole gramicidin S: NBD-PE, *N*-4-nitrobenz-2-oxa-1,3-diazolephosphatidylethanolamine; diI-C_{18}(3), 3,3′-dioctadecylindocarbocyanine iodide; T_m, gel to liquid crystalline phase transition temperature; FRAP, fluorescence recovery after photobleaching; % R, percent recovery; $\tau_{1/2}$, half-time for recovery; w_s, laser spot radius at specimen plane; T_B, time of photobleaching; D, diffusion coefficient; MLV, multilamellar vesicles; DSC, differential scanning calorimetry; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

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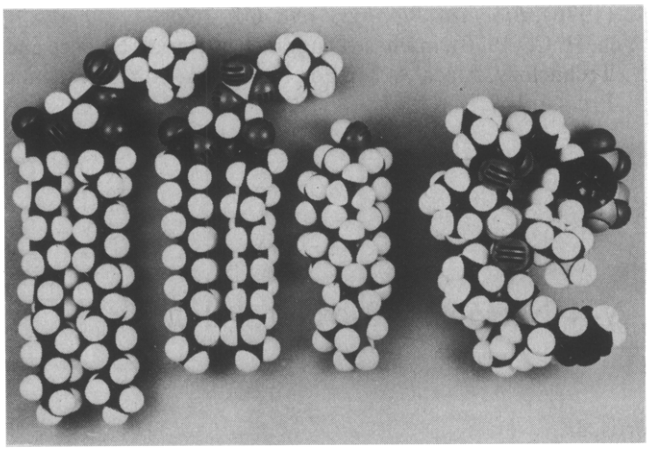

FIGURE 1: CPK models, from left, of distearoylphosphatidylcholine, DMPC, cholesterol, and NBD-GS. The location and orientation of membrane bound NBD-GS is not known (see text for discussion).

Materials and Methods

Lipids. The phospholipids used in this study were the generous gift of Dr. D. Papahadjopoulos and prepared as previously described (Wu et al., 1977). Other lipids, fluorescent labeled phospholipids, and chemicals used are described earlier (Wu et al., 1977). Silica gel H thin-layer plates were obtained from Applied Sciences (State College, Pa.) and activated at 110 °C prior to use.

Preparation of Labeled Gramicidin S. Gramicidin S was obtained from Sigma as gramicidin J. Amino acid analysis of the starting material gave equal molar ratios of proline, valine, ornithine, phenylalanine, and leucine; less than 1% of any other component was detected. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was obtained from Pierce, Rockford, Ill. Labeled GS was prepared by reacting 11 mg of GS dissolved in 0.5 mL of Me_2SO with 2 mg of NBD-Cl in 0.1 mL of Me_2SO in the presence of 5 μL of triethylamine at room temperature for 1.5 h. The NBD-GS was separated from NBD-OH and Me_2SO on a 1 × 52 cm Sephadex LH-20 column eluted with methanol. Amino acid analysis revealed that 60% of the ornithine residues had been modified and the mixture of components was further separated by thin-layer chromatography on silica gel H in chloroform–methanol–glacial acetic acid (7:3:1, v/v). Unreacted GS remained at the point of application while two fluorescent derivatives separated with R_f's of 1.0 and 0.84. Amino acid analysis of the separated components revealed that the component of R_f 1.0 was divalent NBD_2-GS, while the component of R_f 0.84 was monovalent NBD-GS. Examination of the FRAP kinetics of the separated mono- and divalent NBD-GS in multibilayers showed no detectable difference in their diffusion coefficients. Therefore, the unresolved mixture was employed in the lateral diffusion studies; for simplicity, we have referred to this mixture as NBD-GS. Concentrations of the two derivatives were adjusted by NBD absorption at 460 nm based on a molar extinction coefficient of 1×10^4 obtained from Pierce Chemical Co.

Preparation of Lipid Multibilayers. The preparation of the lipid multibilayers was described in detail earlier (Wu et al., 1977). GS was added to the various lipids in organic solvent prior to evaporation. Evaporated lipid–GS films were hydrated in Ca^{2+}, Mg^{2+}-free, phosphate-buffered saline. DPPC samples were prepared inside a hot room at a temperature of 48 °C to ensure that the sample stayed above the phase transition temperature throughout the preparation process. In the course of this study, we observed that FRAP measurements performed on freshly prepared samples would occasionally yield unreproducible results. To avoid this complication, we generally let the sample age for at least 1 day in a sealed chamber of high humidity before performing experiments.

FRAP Measurements. The microscope and methods for performing FRAP measurements have been described in detail previously (Wu et al., 1977; Jacobson et al., 1977). Measurements using different objectives to vary the beam diameter ($2w_s$) indicated that the $\tau_{1/2}$ for recovery scaled as w_s^2 consistent with a diffusion mechanism (Axelrod et al., 1976). Typically, a beam diameter of 8 to 9 μm was employed. Arguments against an appreciable temperature increase during the bleach have been detailed earlier (Wu et al., 1977). In our studies using this instrument, we have noticed that occasionally a neutral density filter, used to attenuate the photobleaching beam for measuring, displaces the measuring beam from the photobleached area. This effect can be checked by bleaching a "hole" in an immobile fluorescent matrix such as a fluorescein Concanavalin A–Sepharose bead (Jacobson et al., 1977), and visually looking for overlap or displacement of this beam from the photobleached "hole". Also, correct alignment is indicated by the measured fluorescence intensity increasing with translation of the bead in any direction after photobleaching. This occasional displacement effect is probably due to the filter acting as a slight optical wedge in the beam. Calculations indicate that very slight nonparallelism of the filter surfaces could deflect the measuring beam by the distances observed. Attenuating filters therefore must be selected with care.

As a measure of precision, the spread in a given set of diffusion coefficients (multibilayer sample at one temperature) generally was within ±10% of the mean value. The accuracy of a given determination of D is more difficult to assess. We have previously quoted an uncertainty of about ±20% based on a 6% uncertainty in the measurement of w_s and a 15% uncertainty in the measurement of $\tau_{1/2}$ (Wu et al., 1977). However, given the observed fluctuations in the laser's capability of holding a perfectly symmetric, Gaussian beam at the specimen plane, we believe that the uncertainty in w_s of ± 20% quoted by Wolf et al. (1977) is probably more realistic. Thus, we would expect the measured D to differ from the true D by about 40%, at most. Some measure of the total system's reproducibility, in which, presumably, errors in w_s are the largest factor, lies in a comparison of our multibilayer results over a period of 2 years. Different investigators using different argon ion lasers (Control 551 vs. Spectra Physics 164-07) have measured values for D on multibilayers of identical composition which generally agree to within 20%.

Differential Scanning Calorimetry. The samples analyzed by differential scanning calorimetry (DSC) were prepared by mixing the desired molar ratios of DMPC or DPPC (in $CHCl_3$) and GS (in absolute EtOH), removal of the solvents under reduced pressure, followed by hydration and vigorous mixing under N_2 at 30 °C for 1 h or more in a buffer containing 100 mM NaCl, 2 mM L-histidine, 2 mM *N*-tris(hydroxymethyl-2-aminoethanesulfonic acid (Tes), and 0.1 mM EDTA. The lipid GS mixtures (5 mol of lipid in 2 mL of buffer) were then collected by centrifugation in an Eppendorf Microfuge for 15 min and transferred to the sample pans (~15 μL). The samples were heated at 5 and 1.25 °C/min (to improve resolution of the peaks) on a Perkin-Elmer DSC-2. Lipid (as phosphate) in the pans was determined by sonicating open the pans in 1.33% deoxycholate, perchloric acid digestion, and phosphate assay as described previously (Papahadjopoulos et al., 1973). Transition enthalpies (ΔH) were determined after measuring area under the excess specific heat curves by paper weighing.

Measuring of Gramicidin S Association with Bilayers. The extent of incorporation of gramicidin S into multilamellar vesicles (MLV) was determined by forming DMPC or DPPC MLV's with 10 mol % added GS. Vesicle-bound GS was separated from the free GS on a sucrose gradient. The prepared MLV's were overlaid on a linear gradient formed from equal volumes of 5.0% sucrose, 1 mM EDTA, pH 7.4, and 20% sucrose, 1 mM EDTA, pH 7.4, total volume 4.6 mL, and centrifuged for 24 h at 35 000 rpm in a SW 50.1 rotor (Beckman) at 25 °C. After fractions were removed from the gradients, GS concentrations were determined with a fluorometric assay (Udenfriend et al., 1972) and phosphate was measured as described in the preceding section.

The DPPC-MLV did not satisfactorily band on the sucrose gradient when centrifuged at 25 °C, so the DPPC (MLV)-GS mixture was resolved on a Bio-Gel A-5m 1 × 25 cm agarose column (Bio-Rad) run at 45 °C and eluted with 0.1 M NaCl. Phosphate and GS concentration was determined on each fraction.

Results and Discussion

On the Location of GS in Lipid Bilayers. Gramicidin S (GS) is a cyclic decapeptide having the following primary structure:

$$[(\text{L-Val-L-Orn-L-Leu-D-Phe-L-Pro})_2]$$

Its secondary structure can be approximated by CPK models, as shown in Figure 1, according to a model based on physical and chemical data in organic solvent (Schwyzer, 1958; Schwyzer & Ludescher, 1968; Kumar et al., 1975). For later reference, the models of cholesterol, DMPC, and DSPC are also given. GS is believed to exert its antibiotic action via the membrane, but its disposition within the membrane with regard to location, conformation, and state of aggregation is not known. Although an earlier report suggests an interfacial location for GS-phosphatidylcholine bilayers (Pache et al., 1972), we believe that it interacts, at least partially, with hydrocarbon region of the bilayer based on several facts. First, GS is highly hydrophobic with the exception of the two ornithine residues and dimeric stacking arrangements can be postulated which shield the charge of these residues. Furthermore, reaction of GS with NBD will neutralize some of the ornithine residues. Secondly, the lateral diffusion of NBD-GS decreases abruptly as the membrane is cooled through its gel to liquid crystalline phase transition temperature (T_m) in a manner identical with fluorescent phospholipid analogues (Wu et al., 1977). The magnitude of D for NBD-GS in bilayers above their T_m is quite similar to that for lipid analogues. Finally, cholesterol can laterally exclude NBD-GS from DMPC and DPPC bilayers suggesting that GS is occupying membrane locations similar to those held by cholesterol. These latter two points will be discussed in detail below. In connection with the last point, early measurements with the spin label, Tempo, showed that its partitioning into bilayer membranes was inhibited by the presence of either cholesterol or GS (Hubbell & McConnell, 1968).

A number of studies described below were performed as a function of the apparent membrane concentration of GS, the word apparent being used to indicate that all of the added GS is assumed to be incorporated. It is an important question whether all of the GS is actually inserted into the multibilayers, especially at high mole fractions of GS. We have approached this question using information from fractionation experiments designed to determine the extent of unincorporated GS, DSC, and fluorescence microscopic studies employing NBD-GS as a marker and the answers, detailed below, depend on the acyl chain composition of the phospholipid bilayer studied.

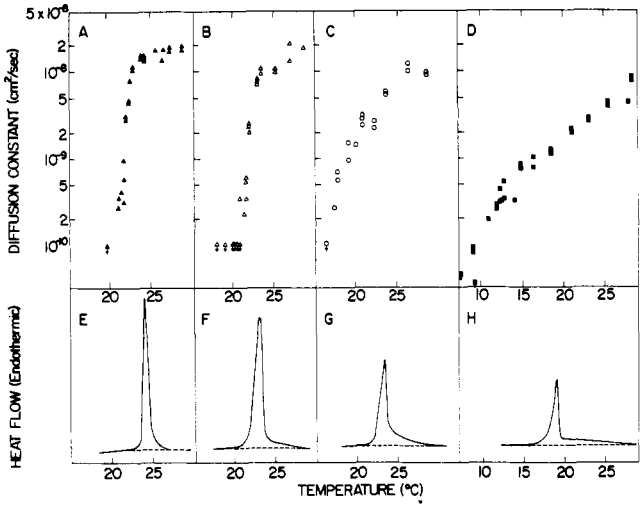

FIGURE 2: [Upper panels (A to D)] Temperature dependence of the lateral diffusion coefficient of NBD-GS in DMPC multibilayers. Molar ratios of GS to DMPC are 1/2500 (A), 1/250 (B), 1/25 (C), and 1/5 (D). $T_B \leq 100$ ms; bleaching power was approximately 7 mW; bleaching beam was attenuated by 10^5 for measuring. ↕ indicates $D \leq$ value on the graph. (Panel E) Thermogram for pure DMPC multilamellar vesicles (no GS present). [Lower panels (F to H)] Thermograms on multilamellar vesicles with the same molar GS/DMPC as those specimens used in panels directly above.

Lateral Diffusion of NBD-GS and DSC Studies of GS in Pure Phospholipid Multibilayers. FRAP measurements were performed on DMPC-GS mixtures at five different molar ratios of DMPC/GS: 25000/1, 2500/1, 250/1, 25/1, and 5/1. In the first two mixtures all of the added GS was NBD labeled. In the latter three mixtures the amount of NBD-GS was maintained at 1/625 (NBD-GS/DMPC), while the remaining GS was unlabeled.

All the samples showed homogeneous fluorescent intensity. Block or domain structures with strong birefrigence in the boundary regions between domains appeared in the polarizing microscope as described before (Wu et al., 1977). The size of the blocks decreased slightly as the GS concentration was increased. In most cases the average dimension of these blocks was of the order of 100 μm. At GS/DMPC = 1/10, the added GS comigrated with DMPC MLV's on a sucrose density gradient suggesting complete incorporation of the peptide into these bilayers. However, at higher GS concentration (2/1, GS/DMPC) a clear separation of GS and lipid occurred and stable multibilayers could not be formed. Such concentrations have been reported to solubilize DPPC (Pache et al., 1972).

Lateral diffusion constants for NBD-GS were measured as a function of temperature for various GS-DMPC mixed multibilayers (Figure 2, panels A through D) and compared with the scanning calorimetry data for similar mixtures of GS and DMPC (Figure 2, panels E through H). Transition parameters determined by lateral diffusion of GS and DSC are compared in Table I. To check the effect of labeling GS with NBD, several DSC experiments were run with pure (NBD-GS)-DMPC mixtures. A representative scan at NBD-GS/DMPC = 1/25 is given in Figure 3, thermogram b (dotted line), and shows qualitatively similar behavior compared with unlabeled GS.

In the comparison of data given in Figure 2, the host lipid liquid crystal melting is measured by DSC while the lateral diffusion of the solute, NBD-GS, is monitored in the FRAP measurement. (Note that Figure 2A through D are semilog plots of D vs. T so that the smaller diffusion constants are magnified.) At low GS concentration (1/25 000, 1/2500, and

TABLE I: DSC and Lateral Diffusion Phase Transition Parameters for GS-DMPC Mixtures.

transition parameter	GS/DMPC mole ratio 0	1/2500	1/250	1/25	1/5
T_m by GS diffusion (°C)[a]		22.5	22.5	23.1	25[c]
transition range (°C)[a]		3.0	2.0	9.75	≈22
		(21 → 23)	(21 → 23)	(16.25 → 26.5)	(8 → 30)
T_m by DSC (°C)	24.4 ± 0.2		23.2 ± 0.2	23.3 ± 0.2	19.1 ± 0.2
transition[b] range (°C)	4.15		7.5	7.75	16.25
	(22.25 → 26.4)		(20 → 27.5)	(21.25 → 29.0)	(11.25 → 27.5)

[a] Transition midpoints estimated by method of Träuble & Overath (1973) on the basis of a linear scale. Transition widths estimated by the temperature (±0.5 °C) at which the curve departs from the behavior characteristic of the gel or liquid crystalline state. [b] Transition widths defined by "liftoff" from estimated base line under endothermic peak. This method was used to deal with the highly asymmetric endothermic peaks at high GS/DMPC. However, by this criterion, the transitions appear considerably broader than when their width is estimated by the width at the half-maximum point or by that temperature interval which encloses 90–95% of the ΔH. [c] T_m is a lower limit since transition may not be complete at the highest temperature of the experiment.

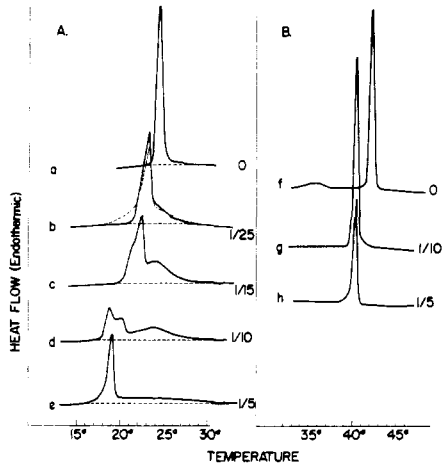

FIGURE 3: Differential scanning calorimetry of GS-DMPC (a-e) and GS-DPPC (f-h) mixtures. Molar ratios of GS added to phospholipid and enthalpy of transition (ΔH) are as follows: (a) DMPC only, 5.4 kcal/mol; (b) 1/25, 5.7 kcal/mol; (c) 1/15, 6.0 kcal/mol; (d) 1/10, 6.2 kcal/mol; (e) 1/5, 7.5 ± 1.0 kcal/mol; (f) DPPC only, 8.5 kcal/mol; (g) 1/10, 9.0 kcal/mol; (h) 1/5, 11.2 kcal/mol. Estimated errors in ΔH are ~0.2 kcal/mol except where stated. For graphically estimated areas under the high, intermediate, and low temperature peaks are respectively (a) DMPC, only, 100%, 0%, 0%; (b) 1/25, 28%, 64%, 8%; (c) 1/15, 39%, 46%, 15%; (d) 1/10, 44%, 24%, 32%; (e) 1/5, ~20%, 0%, ~80%.

1/250 GS/DMPC), the diffusion coefficients went through a clear transition from $D = 1.8 \times 10^{-8}$ cm^2/s at $T \geq 24$ °C to $D \leq 10^{-10}$ cm^2/s at $T \leq 21$ °C. (Data from 1/25 000 mixture is not given but was nearly identical with 1/2500 GS/DMPC.) For these GS/DMPC the midpoint temperatures of the transitions determined from the FRAP data are slightly lower (≈2 °C) than the gel to liquid crystal phase transition region found for DMPC (Figure 2, panel E; Chapman et al., 1967; Hinz & Sturtevant, 1972). This observation suggests that the DMPC surrounding the impurity, NBD-GS, melts before bulk melting of the host crystal occurs; sluggish diffusion may then occur by the NBD-GS melting DMPC as it moves along (Brûlet & McConnell, 1976). These data also suggest that most if not all the NBD-GS is localized within the bilayer since its self diffusion coefficient shows the same sensitivity to a phase transition as do fluorescent lipid analogues (Wu et al., 1977).

As the GS concentration was increased, the transition region was broadened. Figure 2C shows that a molar ratio of 1/25 GS/DMPC the phase transition region of this mixture is extended over a range of about 10 °C between 16 and 26 °C. The diffusion coefficient decreased by a factor of 2 above the transition region and increased by almost the same factor below the transition region compared to lower concentrations of GS. The thermogram for this GS-DMPC mixture (Figure 2G) was also broadened considerably by the appearance of a shoulder on the high temperature side of T_m. At a molar ratio of 1/5 (Figure 2D) the transition was broadened to about 20 °C and the diffusion coefficients varied by a factor of 50 over this range. The transition observed in calorimetry (Figure 2H) was now centered at 19 °C with a long high temperature tail. At the higher GS/DMPC molar ratios (1/25, 1/5) the onset of the change in lateral diffusion begins to substantially precede the onset of lipid melting.

The pronounced asymmetry of the 1/25 GS/DMPC thermogram (Figure 2G) prompted us to examine the calorimetry of GS/DMPC mixtures between 1/25 and 1/5 mol ratio range in more detail; these endotherms, given in the left panel of Figure 3, show a complex behavior ranging from a single highly asymmetric peak at 1/25 to three nearly resolved peaks at 1/10 to a single peak at 1/5, GS/DMPC, with a high temperature tail. The complexity of the GS-DMPC mixtures is in striking contrast to the calorimetric behavior of GS-DPPC mixtures shown in the right panel of Figure 3. Here only a small depression of the T_m and slight broadening of the transition is observed in agreement with the earlier results of Pache et al. (1972). No obvious reason for this marked dependence of transition behavior on chain length has occurred to us from a comparison of CPK models (Figure 1). One possibility is that much of the added GS has separated from the DPPC and is not in the bilayer. Support for this possibility comes from experiments with multibilayers composed of DPPC/GS/NBD-GS (700/70/1) in which large bilayer regions nearly devoid of fluorescence were observed. Secondly, physical separations of MLV-GS mixtures at a 1/10 mol ratio (GS/lipid) reveal that 95 ± 10% of the added GS is associated with the DMPC MLV, while only 45 ± 10% of the added GS is associated with the DPPC MLV. This is additional evidence that at the higher concentrations examined, GS is almost quantitatively incorporated into the DMPC bilayer, although it is excluded to an appreciable extent from the DPPC bilayer.

In the case of the DMPC data, for GS/DMPC mol ratios of 1/25 to 1/5, the thermograms can be graphically resolved into three components whose relative areas are given in the caption to Figure 3. The broad, high melting component in Figure 3 (left panel) could be assigned to free DMPC domains on the basis that the midpoint melting temperature is ≈ 24 °C, the T_m for DMPC. The addition of large amounts of GS, which become "solvated" by DMPC, reduce the size of these domains and the cooperativity in melting. The two lower melting components presumably would be lipid perturbed by adjacent

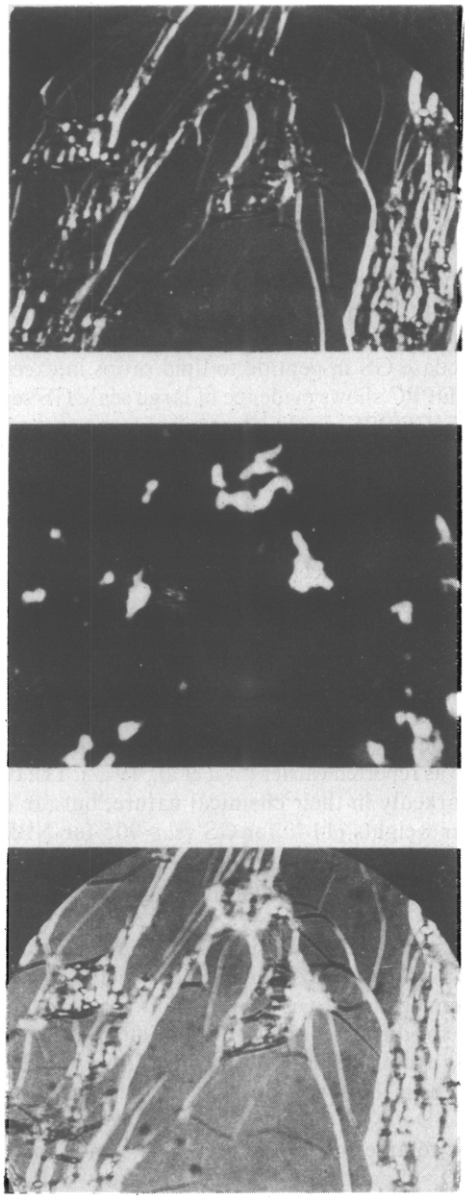

FIGURE 4: Micrographs of NBD-GS in DMPC-cholesterol multibilayers with 50% cholesterol. Scale: 1 cm = 75 μm. (Top) Cross-polarized light microscopy; (middle) fluorescent image using 488-nm excitation; (bottom) image in simultaneous fluorescence and crossed polarizing microscopy.

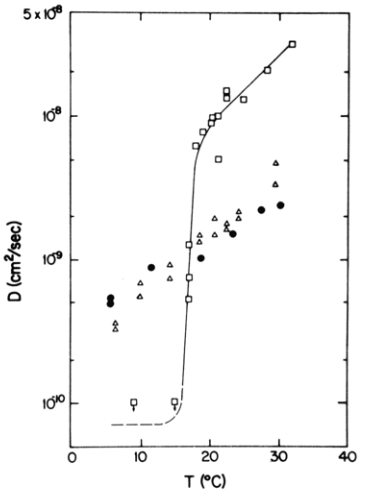

FIGURE 5: Temperature dependence of the lateral diffusion coefficient of NBD-GS in DMPC-cholesterol multibilayers. Cholesterol to DMPC ratios are as follows: 0.44 (△); 0.26 (●); 0.05 (□). FRAP measuring conditions as in Figure 2.

or proximate GS. The changes in the relative magnitude of these components as the GS membrane concentration is increased may then reflect the interplay between a tendency for GS to self-associate and the changing requirements of the layer of lipid required to "solvate" these growing peptide aggregates. However, any conclusive assignment of the endothermic components must await further information on this lipid–peptide interaction.

The lack of such pronounced effects in GS–DPPC mixtures presumably reflects a solubility limit for GS in DPPC. Such a limit could arise, for example, if, at the high mole fractions of GS, association to dimers is generally favored; however, if in a particular bilayer, for example, DPPC, the GS aggregate has the wrong height, the free energy of solution of GS in DPPC would be raised relative to DMPC. On this basis, at the point where oligomer formation should occur, we could expect GS to segregate out of the DPPC bilayer and self-associate in separate patches.

Effect of Cholesterol on the Location and Diffusion of Gramicidin S. The inclusion of cholesterol into phospholipid multibilayers altered both the NBD-GS diffusion coefficient and its location. FRAP measurements were performed on DMPC multibilayers with three different cholesterol contents. Below 10 mol % cholesterol, the uniform fluorescent intensity from the speciman suggested a homogeneous distribution for NBD-GS throughout the multibilayer structure. However, when the cholesterol content was increased to about 20 mol % aggregates of NBD-GS were observed in the boundary regions between "domains", with the fluorescent intensity from the domains accordingly decreased. With 50 mol % cholesterol almost all NBD-GS was segregated into discrete aggregates in certain regions of the domain boundaries as shown in Figure 4. The origin of these NBD-GS rich segregates is not clear. Segregates may be formed by the tendency for NBD-GS to self-associate in the presence of DMPC–cholesterol either as the organic solvent is removed or after hydration. It should also be noted that the formation of these visible aggregates indicates the potential for GS to diffuse as small clusters in the presence of cholesterol. In comparison, at 50 mol % cholesterol in DPPC, part of the NBD-GS was found in the domain boundaries while in 1/1 cholesterol–EPC multibilayers, no domain boundary segregation was observed.

These studies indicate that the ability of a cholesterol–phospholipid bilayer to accommodate impurity molecules is very sensitive to the chain length and unsaturation in the phospholipid component. In this regard, the quenching of chlorophyll a fluorescence when embedded in DMPC–cholesterol bilayers has also been interpreted as a lateral exclusion of chlorophyll from the mixed bilayer (Lee, 1975). Examination of the CPK models in Figure 1 suggests that cholesterol could laterally exclude NBD-GS from the DMPC bilayers by simply occupying its membrane location. Indeed, deuterium magnetic resonance studies indicate that cholesterol uniformly increases the order of EPC acyl chains from the glycerol bridge to carbon 12 (Stockton & Smith, 1976). This study also shows that the longer, mainly C_{18} chains of EPC could allow accommodation of NBD-GS in the region of the bilayer midplane.

Figure 5 shows the temperature dependence of the diffusion constants for NBD-GS in three different DMPC–cholesterol mixtures. With 5 mol % cholesterol in multibilayers, we noticed that diffusion varied substantially between different domains, particularly near the transition region where some domains

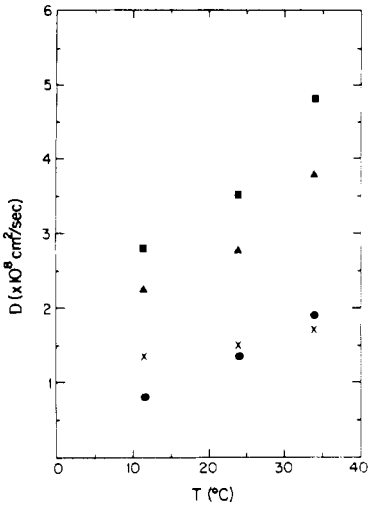

FIGURE 6: Temperature dependence of the lateral diffusion coefficient of NBD-GS in EPC-cholesterol multibilayers. The molar ratios of cholesterol to EPC are 0 (■), 0.1 (▲), 0.5 (X), and 1.0 (●). Each point represents the average of a few independent measurements and bears a random error of about 20% (error bar not shown). NBD-GS/EPC = 1/625; FRAP measuring conditions as in Figure 2.

showed fast recovery while others showed extremely slow recovery. This suggests that the amount of cholesterol may vary among domains. In this regard, phase diagrams of cholesterol–DMPC bilayers indicate solid phase immiscibility below 20 mol % cholesterol and 20 °C with domains consisting of pure DMPC or DMPC–cholesterol in solid solution (Shimshick & McConnell, 1973). The aggregate data show a transition region centered at about 19 °C, somewhat lower than the phase transition temperature of DMPC with no cholesterol. As the cholesterol content was increased to about 21 mol %, the sharp transition disappeared and the diffusion coefficients only varied by a factor of 4 from $D = 5 \times 10^{-10}$ cm^2/s at 6.5 °C to $D = 2.2 \times 10^{-9}$ cm^2/s at 30 °C. Similar behavior was observed at 30 mol % cholesterol. Thus, inclusion of more than 20 mol % cholesterol appears to abolish the phase transition, raising the D for NBD-GS below T_m while decreasing it above T_m. This is consistent with our previous results for lipid lateral diffusion in DMPC bilayers (Wu et al., 1977) and agrees with the general effect of cholesterol on rate processes occurring in bilayers (for review, see Oldfield & Chapman, 1972).

D for NBD-GS in EPC–cholesterol multibilayers is shown as a function of temperature in Figure 6. D decreases as a cholesterol content increases at all temperatures tested. At 50 mol % cholesterol, D decreases by a factor of 2.6. This is consistent with the decrease in both translational and rotational mobility induced by cholesterol for both phospholipid analogues and small, apolar fluorescent probes (see Wu et al., 1977).

Effect of Gramicidin S on Lipid Diffusion. The effect of the polypeptide, GS, on lipid diffusion was studied by measuring the lateral diffusion of NBD-PE, a phospholipid analogue, in EPC multibilayers containing various concentrations of GS. The samples observed in a cross-polarized microscope showed the typical domain structures with the domain boundaries becoming less sharp as the concentration of the GS was increased. The multibilayers showed uniform fluorescence, both when visualized with small amounts of NBD-PE or NBD-GS as the fluorescence probe. However, at the molar ratio of 1/1 (GS/EPC) a visible separation between GS and EPC occurred. At 25 °C and with NBD-PE/EPC = 1/625, no statistically significant change in D of NBD-PE was observed (data not shown) as the apparent GS/EPC was increased to 1/25, but at GS/EPC = 1/5, the D of NBD-PE was reduced by a factor of 1.5 from the value obtained ($\sim 4 \times 10^{-8}$ cm^2/s) in the absence of GS.

Incorporation of GS into Multibilayers. The above studies show that the ability of a bilayer structure to accommodate an impurity such as GS is markedly dependent on both acyl chain composition of phosphatidylcholine as well as the presence of cholesterol. Without cholesterol, a key factor would appear to be how much the hydration temperature for formation of the multibilayer exceeds the transition temperature; this is a measure of the "fluidity" of the bilayer which depends on chain length and degree of unsaturation. Thus, EPC and DMPC can accommodate GS in peptide to lipid ratios in excess of 1/5, whereas DPPC shows evidence of large scale GS segregation at 1/10 GS/DPPC. In the presence of large amounts of cholesterol where the transition is broadened or abolished, chain length appears to determine to what extent NBD-GS will be laterally excluded. That is, at 50 mol % cholesterol, exclusion, judged visually, of NBD-GS is complete in DMPC, partial in DPPC, and does not occur in EPC. It will be interesting to see if the above observations can be generalized to large peptides and membrane proteins.

On the Relationship of Lateral Diffusion to "Membrane Viscosity" and Molecular Size: One prominent finding of this study is that the diffusion constant of NBD-GS closely approaches the values obtained for the fluorescent lipid analogue, NBD-PE as reported earlier (Wu et al., 1977). The two species differ markedly in their chemical nature, but are of similar molecular weights (1140 for GS vs. ≈905 for NBD-PE). In addition, NBD-GS diffusion is limited by phase transitions and cholesterol in a fashion very similar to phospholipid diffusion.

It is instructive to examine existing theoretical relations between the diffusion coefficient, the "membrane viscosity", and the molecular size of the diffusant. The Stokes–Einstein equation for diffusion allows estimation of a membrane viscosity, η_m, from lateral diffusion data, if the membrane is assumed to be a thin, yet isotropic, slab of viscous material in which NBD-GS is dissolved. Assuming that the spherical tumbling volume for GS is about 15 Å in diameter, $\eta_m = kT/6\pi aD \approx 1$ poise, given $D \cong 3 \times 10^{-8}$ cm^2/s for NBD-GS at 300 K in EPC multibilayers. The assumptions of membrane isotropy and sphericity of GS admittedly must introduce some degree of error in this viscosity estimate.

The theory of Saffman (1976) is a more appropriate description of membrane diffusion and would allow calculation of the size of the diffusing entity from the measured D provided an estimate of membrane viscosity were made. As seen below, it can be argued that an estimate for membrane visocisty can be made from the measured lipid diffusion coefficients; however, whether or not GS diffusion data can be analyzed by the Saffman equation remains a moot point. Since the membrane is, theoretically, two dimensional, the Saffman equation (Saffman, 1976: eq 3.10) can be modified by replacing the product of the membrane viscosity and thickness with the surface viscosity, η_s, to yield (Evans & Hochmuth, 1978):

$$D = (kT/4\pi\eta_s)\{\ln(\eta_s/\eta_w a) - 0.577\} \quad (1)$$

where a is the radius of the diffusing cylinder, η_w is the viscosity of the aqueous phase, and k and T have their usual meanings. Given the size of the diffusant, this equation permits calculation of η_s from the measured D if the diffusant is assumed to move only in the two dimensions defining the plane of the membrane so that its motion forces the host amphiphile to move around it. Since collisions in the head-group region appear to dominate the exchange of momentum between the

diffusing entity and the lipid host which is described by the membrane viscosity (see Evans & Hochmuth, 1978), it seems justifiable to apply this treatment to molecules such as labeled phospholipids which penetrate only one monolayer of the bilayer to provide an estimate for η_s. This value is presumably a lower bound because we are considering the bilayer to consist of two uncoupled monolayers. Using the value of $D = 4 \times 10^{-8}$ cm^2/s for NBD-PE diffusion in EPC multibilayers at 25 °C, $a = (70\text{Å}^2/\pi)^{1/2} = 4.7$ Å and $\eta_w = 10^{-2}$ P, a surface viscosity of 5×10^{-7} surface poise can be calculated. If the GS diffusant has $D = 3.5 (\pm 0.5) \times 10^{-8}$ cm^2/s, radii between 5 and 25 Å are compatible with the experimental data; this large range reflects the weak dependence of the diffusion coefficient on radius in eq 1. A great many possibilities for the configuration of the diffusing entity would be consistent with the experimental data. Such configurations might include oligomeric clusters of GS in the form of monolayer or bilayer spanning stacks or a complex consisting of the peptide and adjacent host lipid.

Implications for Biomembranes. The above considerations suggest that even large membrane proteins could diffuse relatively rapidly in fluid bilayers. Indeed, FRAP measurements of "lipophilin" (Moscarello, 1976), the myelin hydrophobic protein, embedded in EPC multibilayers yield diffusion coefficients of approximately 5 to 10×10^{-9} cm^2/s (Derzko & Jacobson, 1978). Similar values were obtained for stearoylated dextrans (mol wt 82 000) adsorbed to black lipid membranes (Wolf et al., 1977), although, in this case, it is not known whether the dextran derivative serves as a model for an integral or peripheral protein. Thus, the slower diffusion of proteinaceous cell surface components ($D \lesssim 4 \times 10^{-10}$ cm^2/s; for example, Edidin et al., 1976; Schlessinger et al., 1977) may not be readily explained on the basis of molecular size or lipid viscosity.

The present and earlier results (Wu et al., 1977) suggest, if a membrane is in the liquid crystalline (fluid) state, lateral diffusion rates of smaller, lipophilic molecules will be relatively insensitive to lipid bilayer composition; for example, cholesterol at 1:1 cholesterol:EPC ratio reduces D by about 60% (Figure 6).

This study also indicates that certain membrane soluble molecules could be at least partially excluded from cholesterol-rich regions of biomembranes. Whether or not cholesterol could partially exclude proteins from domains within the membrane is a moot point. The interaction of lipophilin with 1:1 cholesterol–phosphatidylserine (PS) bilayers does not seem to be reduced compared with pure PS membranes (Papahadjopoulos et al., 1975). On the other hand, fluorescence quenching measurements on membranes having varying phospholipid cholesterol ratios have been interpreted to indicate that increasing cholesterol "vertically" displaces membrane proteins toward the aqueous interface (Shinitzky & Rivnay, 1977). Studies on the interaction of cytochrome *c*, albumin, hemoglobin, lysozyme, and gramicidin A with liposomes using Na^+ permeability changes as an assay have indicated that incorporation of cholesterol into the bilayer generally decreases the interaction of the protein with the bilayer (Papahadjopoulos et al., 1973). It has also been observed that cholesterol must be excluded from the lipid annulus of the Ca^{2+}-ATPase for maximal activity of this membrane enzyme (Warren et al., 1975).

In summary, this work and others in the recent literature describing the measurement of the lateral diffusion of various membrane components in defined model systems should provide an extremely useful background from which data on the dynamics of cellular membranes can be interpreted.

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Conversion of Pre-Proparathyroid Hormone to Proparathyroid Hormone by Dog Pancreatic Microsomes†

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ABSTRACT: In the wheat germ extract and reticulocyte lysate cell-free protein synthesizing systems, the major translation product of bovine parathyroid hormone mRNA was a precursor of parathyroid hormone, pre-proparathyroid hormone and little or no proparathyroid hormone or parathyroid hormone was produced. If dog pancreatic rough microsomes were added to the wheat germ system, a new radioactive protein was synthesized which coelectrophoresed with proparathyroid hormone on acrylamide gels containing sodium dodecyl sulfate and urea–acid acrylamide gels at pH 4. Analysis by Edman degradation of this protein labeled with [^{3}H]lysine revealed lysines at positions 1, 4, and 5 as expected for authentic proparathyroid hormone. Conversion of pre-proparathyroid hormone to proparathyroid hormone occurred only if the membranes were present while pre-proparathyroid hormone was being synthesized. Inhibition of the conversion activity by a nonionic detergent, Nonidet P40, suggested that the proteolytic activity was associated with the membranes. Radioactive proparathyroid hormone but not pre-proparathyroid hormone was protected from digestion by proteolytic enzymes added posttranslationally to the reactions that contained membranes suggesting that proparathyroid hormone had been transported across the microsomal membrane into the lumen of the vesicles. Although analyzed less extensively, the addition of microsomal membranes to the reticulocyte lysate cell-free system is also required for the synthesis of proparathyroid hormone. The fraction of pre-proparathyroid hormone that is converted to proparathyroid hormone in the reticulocyte lysate is about two times that observed in the wheat germ cell-free system. These data demonstrate that, in completely heterologous cell-free systems, pre-proparathyroid hormone can be accurately processed to form proparathyroid hormone by a proteolytic activity associated with microsomal membranes.

In the wheat germ cell-free protein synthesizing system the translational product of parathyroid hormone (PTH)[1] mRNA is pre-proparathyroid hormone (pre-ProPTH) which contains the sequence of PTH and 31 additional amino acids at the N terminus (Kemper et al., 1974, 1976). Pre-ProPTH is essentially the only form of PTH synthesized in cell-free systems that do not contain microsomal membranes, such as the wheat germ system. In intact cells pre-ProPTH is detected in tiny quantities (Habener et al., 1976) and is presumably rapidly converted to proparathyroid hormone (ProPTH) by proteolytic cleavage of 25 N-terminal amino acids. ProPTH is an intracellular precursor which is then converted to PTH by proteolytic cleavage of 6 N-terminal amino acids about 15 min after initial synthesis (Kemper et al., 1972; Cohn et al., 1972). The processing of these larger forms of PTH thus requires two very specific proteolytic cleavages only 6 amino acids apart.

Larger pre-proteins analogous to pre-ProPTH have now been described for many secretory proteins. It has been proposed that the extra sequences at the N terminus (pre-sequence) of these proteins function in the formation of membrane-bound ribosomes and the transport of the secretory proteins through the membrane of the endoplasmic reticulum (Blobel & Sabatini, 1971; Milstein et al., 1972; Blobel & Dobberstein, 1975). The removal of the pre-sequence then would be mediated by enzymes associated with these membranes. In cell-free systems, cleavage of the pre-sequence has been shown to be dependent on the addition of microsomal membrane preparations for myeloma protein (Blobel & Dobberstein, 1975), human placental lactogen (Boime et al., 1977), insulin (Shields & Blobel, 1977), prolactin and growth hormone (Lingappa et al., 1977). The translation of PTH mRNA in the unfractionated Krebs II ascites cell-free system which contains microsomal membranes results in the synthesis of ProPTH as well as pre-ProPTH (Habener et al., 1975). In this paper we show that accurate conversion of pre-ProPTH to ProPTH occurs in the wheat germ and reticulocyte cell-free systems if microsomal membranes from dog pancreas are added to the reaction.

Experimental Procedures

Preparation of Stripped Microsomes. Fresh dog pancreases were chilled and rinsed with 0.9% NaCl and fibrous tissue was removed manually. Five grams of pancreatic tissue in 8 mL of 0.25 M sucrose, 50 mM triethanolamine, pH 7.4 (20 °C), 50 mM KCl, and 5 mM $MgCl_2$ was homogenized with four strokes in a motor driven glass–Teflon Potter-Elvehjen tissue grinder at 150–200 rpm. A postmitochondrial supernatant was prepared by centrifuging the tissue homogenate at 10 000*g* for

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[1] Abbreviations used: PTH, parathyroid hormone; ProPTH, proparathyroid hormone; pre-ProPTH, pre-proparathyroid hormone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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