long-range cooperative interactions in the membrane lipids.

These results depend on how adequately the spin labels $I_{m,n}$ report the overall structure of the labeled membrane. If the labels were localized in small fluid pockets in the membrane one could be misled by the apparent fluidity reported by the labels. It is likely that the labels are reporting an overall structure of the membrane. We have previously shown (Barnett and Grisham, 1972) that the spin labels undergo rapid lateral diffusion in natural membranes and that there can be no substantial local pockets where the label is concentrated. The extent to which the labels themselves are perturbing the membrane structure cannot be answered at this time. However, the amount of label incorporated (*ca.* 1% of the total lipid present) is not large enough to have any effect on the activity of the ATPase.

The precise nature of the role of the lipid-phase transitions in regulating the activity of the $(Na^+ + K^+)$-ATPase has yet to be determined. However, a likely candidate for the step in the enzymatic sequence which is principally affected is the conformational change observed by Post *et al.* (1965).

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Transbilayer Asymmetry and Surface Homogeneity of Mixed Phospholipids in Cosonicated Vesicles†

Daniel M. Michaelson,‡ Alan F. Horwitz, and Melvin P. Klein*

ABSTRACT: Cosonication of equimolar quantities of phosphatidylglycerol (PG) and phosphatidylcholine (PC) results in bilayered vesicles the outer surface of which contain, on the average, twice as many PG as PC molecules. Within the surface these two lipid classes are not spatially segregated into "patches." These results were obtained by exploiting the effects of paramagnetic ions on the proton and phosphorus nuclear magnetic resonances. The ^{31}P resonances of PG and PC sonicated separately have different chemical shifts and broaden differently upon addition of Mn^{2+}. At Mn^{2+} concentrations less than 10^{-4} M, these ions do not permeate the vesicles, permitting a distinction of the signals originating on the outer surface from those on the inner surface. For pure dispersions of PG and of PC, Mn^{2+} and Eu^{3+} reside closer to the phosphate than to the choline $N(CH_3)_3^+$ protons and the residence time of Mn^{2+} is short, less than 10^{-4} sec. The integrated and asymmetric arrangement of the phospholipid molecules in the cosonicated dispersions is discussed in the context of the structure and biosynthesis of biological membranes.

Phospholipids are a major constituent of many cellular membranes. These molecules are present in several classes, each defined according to its polar head group, and within each class there is great diversity in the fatty acid composition. The variability in the fatty acid composition has been investigated extensively, and its physiological significance is beginning to be understood (Cronan and Vagelos, 1972; Singer and Nicolson, 1972); however, little is known about the function of the variability in the head-group composition. Nevertheless the observations that different membranes often contain different phospholipid classes (Rouser *et al.*, 1968; Kates and Wassef, 1970) and that in some cases cells alter their membrane phospholipid composition in response to changes in external parameters (Op den Kamp *et al.*, 1969, 1971; Haest *et al.*, 1972) suggest that this variability is of structural and physiological importance.

The majority of the phospholipids are believed to be ar-

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ranged in a two-dimensional fluid bilayer (Singer and Nicolson, 1972). However, the spatial organization of the phospholipids within the bilayer is not certain; neither are the factors affecting it understood. Previous work by Bretscher (1972) and by Caspar and Kirshner (1971) suggests that different lipids may be preferentially partitioned between the inner and outer faces of the membrane, thus rendering it asymmetric. The demonstration that specific phospholipids are required for the activation of certain membrane proteins (Steck and Fox, 1972) may suggest that within the plane of the membrane these molecules are arranged in a nonrandom heterogeneous pattern.

In order to gain a further understanding of some of the factors which affect the arrangement of phospholipids in cellular membranes, we studied their organization in a model system, *i.e.*, sonicated phospholipid dispersions (Atwood and Saunders, 1965). In this paper we present our observations regarding the arrangement of a zwitterionic phospholipid–phosphatidylcholine, and an anionic one–phosphatidylglycerol, within and between the monolayers of the bilayered sonicated vesicles. We have employed the phosphorus and proton magnetic resonance (pmr) spectra of the sonicated dispersions and the effects thereon of paramagnetic ions (*e.g.*, Mn^{2+}, Eu^{3+}) to study differentially the organization of the phospholipids.

Materials and Methods

Egg-yolk phosphatidylcholine (PC)[1] was extracted and purified following the procedures of Singleton *et al.* (1965). Phosphatidylglycerol (PG) was synthesized enzymatically from PC and distilled glycerol employing the alcohol transferase activity of phospholipase D. The enzyme was purified from Savoy cabbage by the procedure of Yang (1969) except that only the first three steps—*i.e.*, preparation of crude extract, heat coagulation, and acetone precipitation—were employed. The enzyme was assayed as previously published (Yang, 1969) with the exception that the reaction was terminated with citric acid (0.1 M final concentration) and that the ether used was distilled over P_2O_5. The resulting phospholipids, PG, phosphatidic acid, and PC were separated on thin-layer chromatographic (tlc) plates which were prepared by coating glass plates with a 0.5-mm thick suspension of silica gel (Adsorbosil 5 obtained from Applied Science) in a solution of methanol–water (1:1) containing 0.5 M oxalic acid. The plates were developed in chloroform–methanol–concentrated HCl (87:13:0.5, v/v) and phospholipid spots were visualized with iodine vapors, scraped, and then analyzed for phosphorus content (Yang *et al.*, 1967). With our conditions, the reaction yield was approximately 60% PG, 40% phosphatidic acid, and a trace amount of PC. PG, phosphatidic acid, and PC were purified from this mixture by DEAE-cellulose column chromatography, employing an elution scheme similar to that described by Rouser *et al.* (sequence 3 in Rouser *et al.*, 1969), except that elution with chloroform–methanol (9:1, v/v) and acetic acid was omitted. After elution from the column, PG was dissolved in chloroform and converted to the Na salt by two extractions with 0.5 M aqueous Na_2SO_4; it was stored as the Na salt in chloroform under argon at −20°. The purified PG gave a single spot on tlc and was further identified by its pmr spectrum.

Sonicated PC and PG vesicles in 50 mM sodium borate buffer (pD = 8.0), containing 150 mM NaCl and 10^{-6} M EDTA were prepared as previously described (Horwitz and Klein, 1972). Both phospholipids yielded clear dispersions on sonication (Papahadjopoulus and Ohki, 1970; Atwood and Saunders, 1965). Cosonicated PG:PC vesicles were prepared similarly from equal amounts of PG and PC. In the experiments employing Mn^{2+} and Eu^{3+}, small volumes of the above buffer containing $MnCl_2$ or $EuCl_3$ were added to the phospholipid dispersions after sonication. The sample was vortexed gently during the addition of the paramagnetic ions in order to obtain rapid mixing. After every experiment the phospholipids were extracted by the Bligh and Dyer method, as described by Ames (1968), and were shown by tlc to each give a single spot.

Pmr spectra were recorded at 20° on a Varian HR-220 spectrometer with Me_4Si as an external standard. Phosphorus magnetic resonance measurements were made in a pulsed Fourier mode at 24.3 MHz, as described elsewhere (Horwitz and Klein, 1972), with an external standard of a saturated pyrophosphate solution at pH 9.0.

Results

Aqueous Sonicated Dispersions of PG and PC Are Distinguishable. COMPARISON OF PG AND PC PHOSPHORUS AND PROTON MAGNETIC RESONANCE SPECTRA. Sonicated PC and sonicated PG dispersions have characteristic and distinguishable phosphorus and proton magnetic resonance spectra which are sensitive to the dynamics and environment of the phospholipids (Horwitz, 1972; Horwitz *et al.*, 1973). The phosphorus magnetic resonance spectra of sonicated PC and of sonicated PG, shown in Figure 1a, each contain a single peak. The PC phosphorus line width (19 ± 1 Hz) is narrower than the corresponding PG line width (30 ± 2 Hz), and the two resonances have different chemical shifts: PC is shifted 0.93 ppm (22.5 Hz) to higher field from PG. The pmr spectra of sonicated PC and sonicated PG are likewise distinguishable. Sonicated PC dispersions have an intense choline $N^+(CH_3)_3$ proton peak which is absent in the corresponding PG spectra, while PG dispersions have an additional peak which is absent in the PC dispersions. This peak is shifted 3.7 ppm to lower field from Me_4Si and is 27 ± 2 Hz wide; its relative area and chemical shift are consistent with the assignment that it arises from the nonexchangeable protons of carbons-2 and -3 of the head-group glycerol.

PARAMAGNETIC IONS INTERACT DIFFERENTLY WITH SONICATED DISPERSIONS OF PC AND PG. The effects of paramagnetic ions, *e.g.*, Mn^{2+} and Eu^{3+}, on nuclear magnetic resonances are determined by the nature of the ion and the structure and environment of the molecule with which the ion interacts (Appendix 1). The effects of Mn^{2+} on the phosphorus and proton magnetic resonance spectra of sonicated PC and sonicated PG are different. The extent of broadening of the resonances of sonicated PC by Mn^{2+} is shown in Figure 2. The addition of increasing concentrations of Mn^{2+} to sonicated PC dispersions broadens and hence decreases the amplitude of the phosphorus and choline $N^+(CH_3)_3$ proton resonances. At 10^{-5} M Mn^{2+} each of these resonances contains two components, one broadened and one unaltered. At higher Mn^{2+} concentrations (approximately 10^{-4} M) a plateau is reached where additional increase in Mn^{2+} concentrations causes no further decrease in peak height. The line width and chemical shift of the residual phosphorus and choline $N^+(CH_3)_3$ proton resonances are equal to those of the original peaks, whereas their areas are 37% of the initial peak areas.

[1] Abbreviations used are: PC, phosphatidylcholine; PG, phosphatidylglycerol.

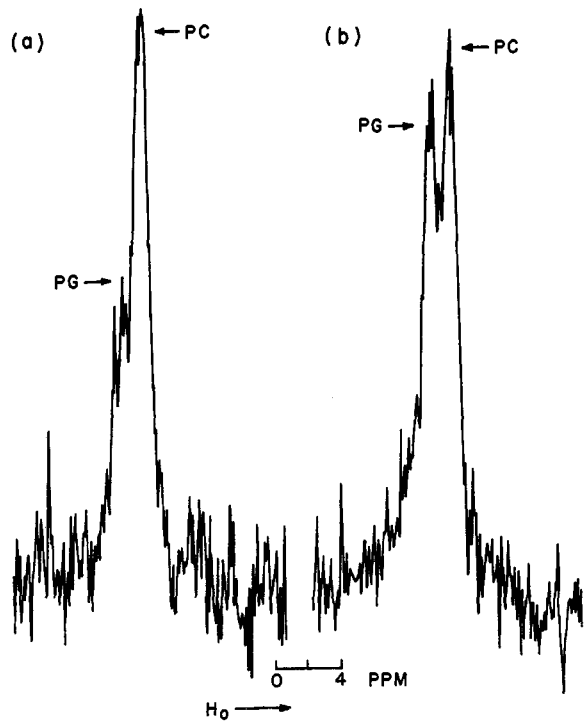

FIGURE 1: Comparison of the phosphorus magnetic resonance spectra of PG and PC in sonicated dispersions: (a) ^{31}P spectrum of a mixture of 30 mM sonicated PG and 30 mM sonicated PC; (b) ^{31}P spectrum of the same sample after cosonication.

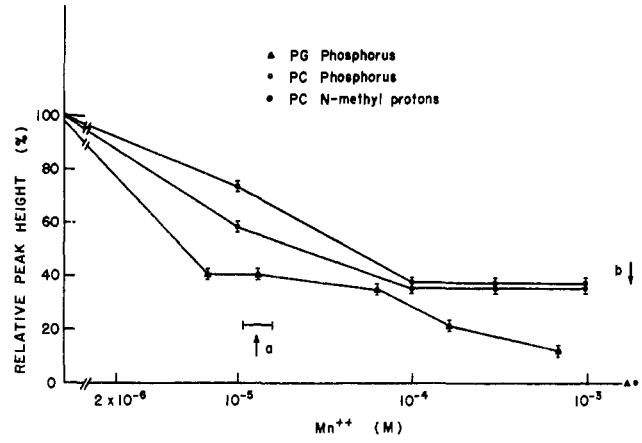

FIGURE 2: The change in peak height relative to the original resonance *vs.* $MnCl_2$ addition to sonicated PC and to sonicated PG dispersions. For PG the phosphorus magnetic resonance line width at the plateau was narrower than the initial resonance. This difference was accounted for by normalization of the phosphorus magnetic resonance peak height (see text). Thus, for all the graphs the per cent peak height at the plateau is equal to the detectable fraction of the original intensities. The concentration of the vesicles is indicated by (a), and the signal intensities after resonication in the presence of Mn^{2+} by (b).

Resonication of the vesicles in the plateau regime (*e.g.*, in the presence of 10^{-3} M Mn^{2+}) results in broadening, beyond detection, of all of the phosphorus and choline $N^+(CH_3)_3$ proton resonances. At these concentrations there is no detectable effect of Mn^{2+} on the intensity and line widths of the fatty acid proton resonances.

Our interpretation of the data agrees with that of Bystrov *et al.* (1971). Sonicated PC vesicles are impermeable to Mn^{2+} ions, and hence the Mn^{2+} broadens only the resonances of nuclei which face the "outside" of the vesicle and are present at or very near the polar region. The plateau therefore arises when sufficient Mn^{2+} is added to broaden, beyond detection, the resonances which arise from the "outside" nuclei. The magnitude and equality of the plateaus is in accord with the size of the single shelled sonicated PC vesicles and the bilayer's thickness (Huang, 1969). Thus resonication in the presence of Mn^{2+} results in the introduction of the ion into the internal aqueous phase of the vesicles, and the consequent broadening of the "inside" as well as "outside" phosphorus and choline $N^+(CH_3)_3$ proton resonances.

The effect of Mn^{2+} on the phosphorus magnetic resonance spectra of sonicated PG is shown in Figure 2. The addition of 7×10^{-6} M Mn^{2+} (less than one ion per vesicle), which is the lowest concentration examined thus far, to sonicated PG dispersions broadens beyond detection approximately 60% of the original phosphorus resonance; no further change is observed on increasing the Mn^{2+} concentration up to approximately 10^{-4} M. The similarity in the intensities at the plateaus of the PC and PG ^{31}P resonances suggests that both types of vesicles have a similar ratio of "outside" to "inside" molecules, that sonicated PG vesicles are mostly single shelled, and that their size and shape are similar to those of sonicated PC vesicles. The phosphorus magnetic resonance line width of sonicated PG in the plateau regime (14 Hz) is narrower than that in the absence of the metal ion (30 Hz). By contrast, as noted above, the width of the phosphorus magnetic resonance of PC vesicles is the same at the plateau of $[Mn^{2+}]$ as it is prior to the addition of the ion. We have observed that the position of the PG phosphorus resonance at the plateau is shifted by 0.32 ppm (8 Hz) to higher field than the initial resonance. A plausible explanation of this observation is that the resonance position of the "inside" and "outside" PG phosphorus resonances differ by ~0.5 ppm. (The phosphorus magnetic resonance chemical shifts of phosphates and phosphate esters are dominated by the state of protonation, which suggests that the separation between the "inside" and "outside" phosphorus magnetic resonance may be enhanced by a pH gradient across the vesicle as well as observation at higher magnetic fields.)

Mn^{2+} concentrations higher than 10^{-4} M result in a gradual decrease in the PG phosphorus peak height (Figure 2); however, the width of the residual line is unaltered. At these Mn^{2+} concentrations the sample becomes turbid and a decrease in the gross intensity of the pmr spectrum is observed, suggesting that the decrease in the intensity of the phosphorus resonance is due to ion leakage and vesicle precipitation. This interpretation is supported by observations (Papahadjopoulus and Ohki, 1970; Ohki, 1972). that asymmetric addition of bivalent ions to sonicated phospholipid vesicles and to black membranes composed of anionic phospholipids induces their leakage and breakage.

A comparison of the effects of Mn^{2+} on sonicated PG and sonicated PC suggests that the interaction of Mn^{2+} with PG is stronger than that with PC. This conclusion is based on the observations that, unlike with PC, Mn^{2+} detectably broadens the fatty acid methylene proton resonances of PG, and that the phosphorus peak of PG reaches its plateau at significantly lower Mn^{2+} concentrations than does the PC phosphorus resonance. These differences are not unexpected, since Mn^{2+} broadening depends on the binding constant and the nature of the complexes formed between Mn^{2+} and the phospholipids (see Appendix 1). As is shown schematically in Figure 3, we can expect that the structural and organizational differences between PG and PC could cause their resonances to be differentially broadened by Mn^{2+}.

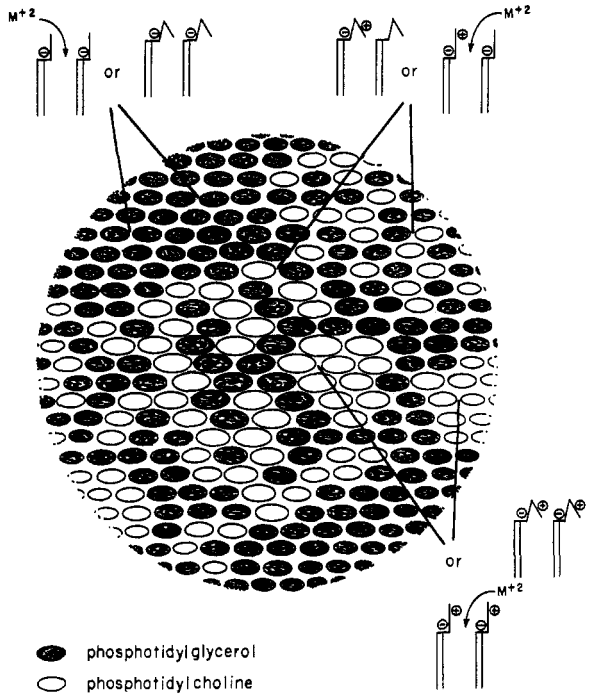

FIGURE 3: A perspective view of a bilayer illustrating possible spatial arrangements of the two different classes of phospholipids and some different possible interactions between neighboring phospholipids in the presence and absence of metal ions.

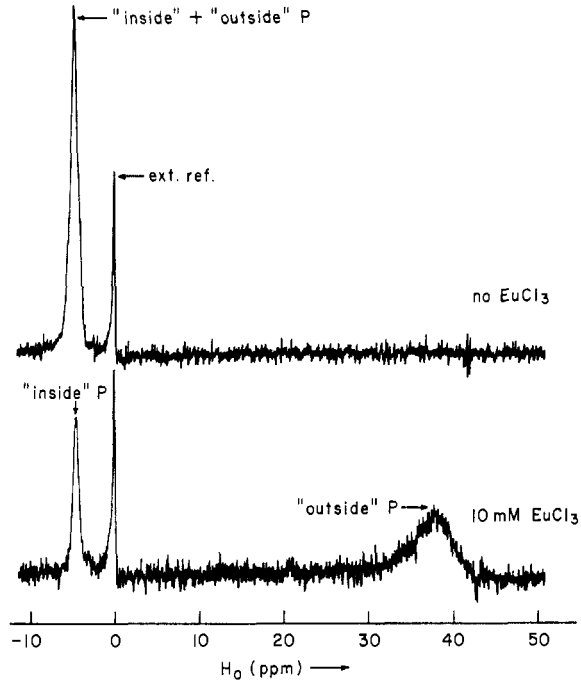

FIGURE 4: ^{31}P spectrum of 50 mM sonicated PC, before and after the addition of 10 mM $EuCl_3$. The external reference is pyrophosphate at pH 9.0. "Outside" P corresponds to those phosphorus atoms in the outer monolayer, while "inside" P corresponds to those in the inner monolayer.

Another useful paramagnetic ion is Eu^{3+} which, unlike Mn^{2+}, primarily shifts nuclear magnetic resonances when the chemical exchange between bound and free rare earth ion is rapid (Eaton and Phillips, 1965). This ion also has been employed by Bystrov *et al.* (1971), who showed that when added to sonicated PC it shifts to higher fields the choline $Na(CH_3)_3$ proton resonance which originates from PC molecules present on the "outside" of the vesicle. We have extended these studies and investigated the effect of Eu^{3+} on the pmr and also on the phosphorus magnetic resonance spectra of sonicated PC and PG dispersions. With sonicated PC we found that Eu^{3+} shifts part of the phosphorus resonance (Figure 4) and part of the choline $N^+(CH_3)_3$ proton resonance to higher fields. In both instances the unshifted component has the same line width as the original peak, and it retains approximately 37% of the area of the original peak, whereas the shifted component is broadened and contains approximately 63% of the total area. The extent of the shifts, shown in Figure 5, was much greater for the phosphorus than for the choline $N^+(CH_3)_3$ peaks. Resonication in the presence of Eu^{3+} results in the introduction of Eu^{3+} into the vesicle's inner aqueous phase (Bystrov *et al.*, 1971), and therefore the resonicated vesicles have single phosphorus and choline $N^+(CH_3)_3$ proton peaks which have the same line width and shifts as those of the shifted resonances prior to resonication.

The interaction of Eu^{3+} with sonicated PG was quite different from its interaction with sonicated PC. By contrast with PC, the addition of Eu^{3+} to sonicated PG dispersions did not significantly shift but rather broadened the phosphorus peak. We attribute this broadening to a lowered rate of exchange between bound and free rare earth, thus lending further support to the conclusion that the metals bind more strongly to PG than to PC. The extent of broadening paralleled the Eu^{3+} concentration and resulted in broadening beyond detection of the total phosphorus peak at 1 mM Eu^{3+}; at approximately 5×10^{-4} M Eu^{3+}, the vesicles began to leak and precipitate. In view of these observations, we chose to employ Mn^{2+} as out initial probe in investigating the organization of PG and PC in cosonicated vesicles.

PG and PC within the Outer Monolayer of a Cosonicated PG:PC Vesicle Are Not Segregated. In cosonicated PG:PC vesicles, the PG and PC phosphorus resonances have similar line widths (approximately 21 Hz). This contrasts with what is observed with pure PC and pure PG vesicles mixed together, as is shown in Figure 1. The narrowing of the PG phosphorus resonances upon cosonication indicates that our cosonicated vesicles each contain PC and PG molecules. This assertion is in accord with previous experiments (Bangham, 1968) which have shown that cosonication of a mixture of phospholipids results in mixed vesicles. Further, the narrowing of the PG phosphorus resonance line to a width similar to that of PC suggests that in the cosonicated vesicles PG and PC do not reside in segregated environments, but rather are dispersed within the vesicle's monolayers.

A second line of evidence suggesting a non-"patched" arrangement within the outer monolayer, is the nature of the broadening by Mn^{2+} of the phosphorus and choline $N^+(CH_3)_3$ proton resonances. From the slopes shown in Figure 6 we observe that in cosonicated vesicles the peak heights of the PG and PC phosphorus resonances decrease similarly with increasing Mn^{2+} concentrations, and that they are intermediate to those observed in the respective pure PC and pure PG experiments (Figure 2). This finding is incompatible with the existence, within the outer monolayer, of large aggregates of PG and PC (see Appendix 2). The data shown in Figure 7, that the choline $N^+(CH_3)_3$ proton peak reaches its plateau at lower Mn^{2+} concentrations in cosonicated vesicles than in pure PC vesicles, further supports this conclusion.

In Cosonicated PG:PC Dispersions PG and PC Are Distributed Asymmetrically between the Inner and Outer Mono-

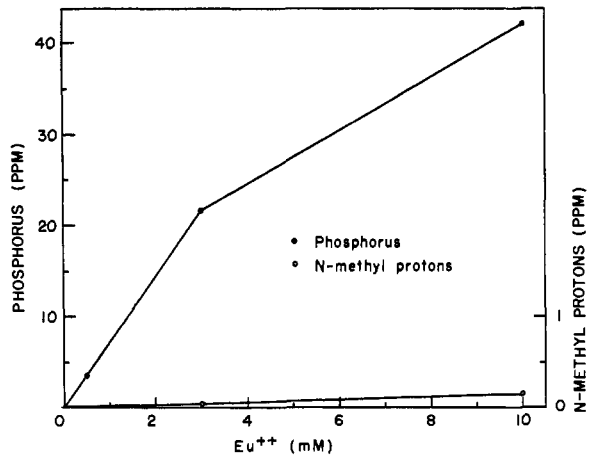

FIGURE 5: Eu^{3+}-induced chemical shift of the "outside" ^{31}P and choline *N*-methyl proton resonances of sonicated PC (50 mM).

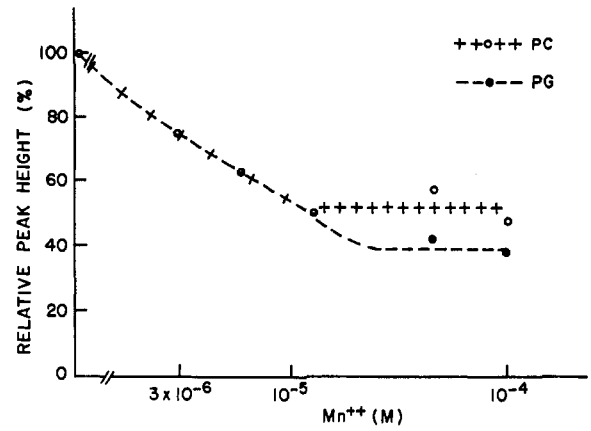

FIGURE 6: The change in the peak height relative to the original ^{31}P resonance *vs.* $MnCl_2$ addition to 60 mM cosonicated PG:PC (1:1) dispersions.

layers. Analogously to what is observed with pure dispersions (Figure 2), the gradual broadening of the phosphorus resonances of PG and PC, by increasing Mn^{2+} concentrations, is followed by a plateau which extends from 10^{-5} M to approximately 10^{-4} M Mn^{2+}. (At higher concentrations there is further decrease in the intensity of the phosphorus resonances, probably resulting from vesicle leakage and precipitation.) Figure 6 shows that the amplitude of the PC peak at the plateau is higher than that of PG. Since these residual resonances have similar line widths which are the same as those of the original peaks, and because the sum of their areas is 40% that of the unbroadened PG and PC resonances, we can conclude that the "inner" monolayer contains more PC than PG. The error in the intensity of the PG phosphorus resonance at the plateau is relatively large, due to the smaller amount of PG present on the "inside"; and consequently only a qualitative statement can be made concerning the asymmetry of the vesicles.

A more quantitative measure of the degree of asymmetry is provided by the pmr results shown in Figure 7. The plateau levels of the choline $N^+(CH_3)_3$ proton resonances in cosonicated PG:PC and in pure PC vesicles are substantially different. For the cosonicated vesicles, at the plateau approximately 40% of the original choline $N^+(CH_3)_3$ proton resonance is broadened while in pure PC dispersions about 63% is affected. The "outside" monolayer in the cosonicated vesicles contains 60% of the total PG plus PC molecules; because only 40% of the PC is on the outside, we thus obtain that in cosonicated vesicles there are twice as many PG as PC molecules facing the outside. These results cannot be accounted for by the possibility that cosonication resulted in a heterogeneous population of symmetric vesicles. We thus conclude that the individual vesicles are asymmetric, and that on the average their outer monolayer contains twice as many PG as PC molecules.

Discussion

Our data suggest that PG and PC are distributed asymmetrically between the inner and outer halves of the bilayered cosonicated dispersions, and that within the outer monolayer (and possibly the inner monolayer) these molecules are not arranged in large aggregates which contain significantly more molecules in their center than on their boundaries. We may further infer from our Mn^{2+} experiments that at the concentrations employed, the ion does not induce the formation of such "patches." The absence of large segregates may be explained by the fact that PG is negatively charged while PC is zwitterionic; due to the electrostatic repulsion between the PG molecules, their aggregation is energetically unfavorable. Although a conclusion regarding the precise arrangement of these phospholipids in the bilayer cannot as yet be made, a plausible arrangement is one in which the PG molecules are separated by the PC molecules, thus minimizing the electrostatic repulsion between the negatively charged PG. A similar pattern has been suggested for the arrangement of acidic and zwitterionic phospholipids in monolayers (Dawson, 1968). However, under suitable conditions different phospholipids may segregate. For example, those with fatty acids of different lengths and/or degrees of unsaturation may be separated at the appropriate temperatures into different phases (Ladbrooke and Chapman, 1969; A. F. Horwitz, unpublished results) and, further, appropriate concentrations of polyvalent ions may induce the segregation of differently charged phospholipids.

The observed asymmetry of the cosonicated vesicles is most

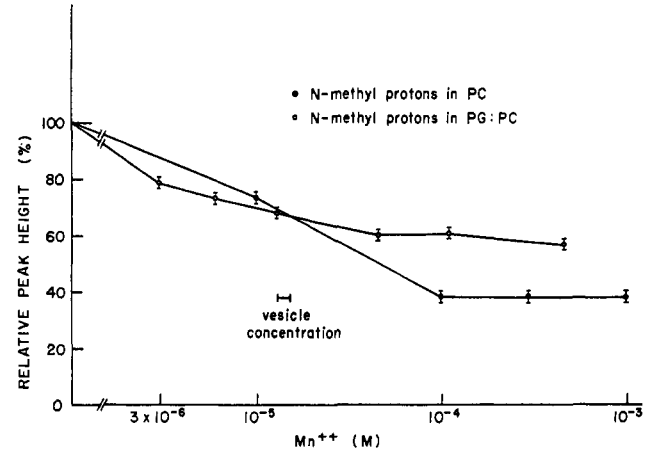

FIGURE 7: The change in the relative peak height of the PC *N*-methyl proton resonance *vs.* $MnCl_2$ addition to sonicated PC and to cosonicated PG:PC (1:1) vesicles. For the cosonicated vesicles the PC *N*-methyl peak at the plateau (Mn^{2+} concentrations higher than 4×10^{-4} M) was broader (14 Hz) than the original peak (9 Hz). The data were normalized to account for this difference; thus for both plots the per cent peak height at the plateau is equal to the detectable fraction of the original intensity.

likely due to the small radius of curvature of the vesicles and to the difference in the charge of the PG and the PC head groups. That is, since the cosonicated vesicles are of a size similar to that of the PC vesicles, *i.e.*, 200–300 Å (see Results section), the head groups of the molecules in the inner monolayer are packed more tightly than those in the outer monolayer. As a consequence, the electrostatic repulsion between the negatively charged PG molecules will be reduced in an asymmetric arrangement with more of the PG present on the outside. The asymmetry of the vesicles is not induced by Mn^{2+}, since the distribution of PG and PC across the bilayer was unaltered by increasing Mn^{2+} concentrations (see Figures 6 and 7).

It is likely that the phospholipids are partitioned asymmetrically between the inner and outer faces of some biological membranes and that they are in part segregated within the plane of the membranes. Bretscher (1972) has proposed that in red blood cells the phospholipids are distributed asymmetrically between the faces of the membrane, and the observation that specific phospholipids are required for restoring the activity of several isolated membrane proteins (Steck and Fox, 1972) may be interpreted to suggest that in the membrane some of the phospholipids are segregated. The presumed asymmetric organization of the phospholipids may be of physiological importance; in particular, it may provide the proper matrices for enzymes which are located only on one side of the membrane (Douce *et al.*, 1973; Fressenden-Raden and Racker, 1971). The assumed asymmetric and patched arrangement of the phospholipids may be determined by several processes. It might arise thermodynamically by the interactions between the membrane components, particularly those between the phospholipids and the proteins and between phospholipids differing in fatty acids and head groups, it might arise biosynthetically, or it might arise by a combination of both.

With reservations, the conclusions obtained from our experiments may be applicable to the problem of the organization of biological membranes. Some membranes such as the highly convoluted mitochondrial inner membrane and the membranes of neurotransmitter vesicles have radii of curvature similar to those of the sonicated vesicles. The size and shape of such membranes may affect the distribution of differently charged phospholipids between the two faces of the membrane. Also, the electrostatic potentials present across many cellular membranes may modify the distribution of dissimilar phospholipids across the membrane. (Alternatively, a spontaneous transmembrane potential can arise from an asymmetric arrangement of phospholipids with differently charged head groups.) We further infer that to the extent that acidic and zwitterionic phospholipids with similar fatty acids are segregated in biological membranes, such an arrangement does not arise spontaneously, but rather it is induced by other factors such as the membrane proteins and polyvalent cations.

A useful approach for studying the problem of the organization of phospholipids in biological membranes may be to investigate the effect of paramagnetic ions on their phosphorus and proton magnetic resonance spectra. Phosphorus magnetic resonance is particularly suitable because in membranes the major phosphorus containing molecules are the phospholipids, and in addition, the observation that PG and PC phosphorus resonances have different chemical shifts and the likely possibility that other head groups also have different shifts, presents the potentiality of differentially studying the phospholipids in intact cellular membranes. Since the biological membranes examined thus far have broad phosphorus resonances (Davis, 1972), line-narrowing techniques (Sarles and Cotts, 1958; Mehring *et al.*, 1971) and shift reagents including lanthinide and transition groups ions might be useful for these studies.

Acknowledgments

D. M. M. thanks Dr. M. Tomkiewicz for many stimulating and informative discussions.

Appendix I

The broadening by Mn^{2+} of the resonances of nuclei present on the outer monolayer of sonicated phospholipid dispersions can be treated similarly to the case of aqueous solutions of Mn^{2+}. Equation 1 derived by Swift and Connick (1962) describes the broadening of the water proton resonance in a dilute Mn^{2+} solution.

$$1/T_2 - 1/T_{2_0} = pq/(T_{2m} + \tau) \quad (1)$$

where T_2 and T_{2_0} are respectively the proton transverse relaxation times in the presence and absence of Mn^{2+}, T_{2m} is the transverse relaxation time of the nuclei when bound to Mn^{2+}, τ is the lifetime of the water–Mn^{2+} complex, q is the solvation number of Mn^{2+} in the complex, and p is the molar ratio of Mn^{2+} to water protons. For the derivation of this equation it was assumed that the protons are in two environments, bound to Mn^{2+} and unbound in the bulk solvent, and that the Mn^{2+} concentration is much less than the proton concentration. In applying this equation to the nuclear resonances of phospholipids arranged in sonicated dispersions, two additional points should be noted. First, the Mn^{2+} can be bound either to the phospholipids or free in the bulk solution, and second, the phospholipids are arranged in a two-dimensional array which may affect the value or values of q.

Mn^{2+} may form several types of complexes with the sonicated dispersions: each complex is characterized by the number of phospholipid molecules which are bound to the ion, j, and the number of water molecules, i, which are displaced by the phospholipids from the first coordination sphere of Mn^{2+}. We designate $[Mn^{2+}PL_i^j]$ as the concentration of complexes where j phospholipid molecules are bound to Mn^{2+}, and a fraction i of these molecules is complexed in the first coordination sphere of the ion. In addition, the total concentrations of Mn^{2+} and the outside phospholipids are represented by $[Mn^{2+}]_0$ and $[PL]_0$. Let us assume that a given phospholipid does not exchange directly between two Mn^{2+} complexes (this assumption is justified since in our experiments $[Mn^{2+}]_0 << [PL]_0$); then eq 1 takes the form

$$\Delta \equiv 1/T_2 - 1/T_{2_0} = \sum_{i,j} \frac{[Mn^{2+}PL_i^j]}{[PL]_0} \left(\frac{i}{T_{2m_i}^j + \tau_i^j} + \frac{j - i}{T_{2n_i}^j + \tau_i^j} \right) \quad (2)$$

where τ_i^j is the lifetime of the $Mn^{2+}PL_i^j$ complex, $T_{2m_i}^j$ is the transverse relaxation time of the resonances which arise from the i molecules which are bound to the first coordination sphere of Mn^{2+}, and $T_{2n_i}^j$ is the transverse relaxation time of the resonances which arise from the $j - i$ molecules in the complex which are not bound in the first coordination sphere of the ion. The summation extends over all possible complexes.

We define apparent equilibrium constants

$$K_i^j = \frac{[\mathrm{MnPL}_i^j]}{[\mathrm{Mn}^{2+}]_f[\mathrm{PL}]_f} \quad (3)$$

where $[Mn^{2+}]_f$ and $[PL]_f$ are respectively the concentrations of the free Mn^{2+} and the free outside phospholipids. By combining eq 2 and 3, we get eq 4.

$$\Delta \equiv 1/T_2 - 1/T_{2_0} = \sum_{i,j} \frac{K_i^j[\mathrm{Mn}^{2+}]_0}{1 + [\mathrm{PL}]_0\left(\sum_{i,j} K_i^j\right)}\left(\frac{i}{T_{2\mathrm{m}i}^j + \tau_i^j} + \frac{j-i}{T_{2\mathrm{n}_i}^j + \tau_i^j}\right) \quad (4)$$

Let us assume that all the Mn^{2+} complexes contain q phospholipid molecules, that the complexes have similar lifetimes, τ, and that in the complexes the transverse relaxation times of similar nuclei are equal and designated by T_{2m}. If now we define an effective equilibrium constant

$$K_{eff} = \frac{[\mathrm{Mn}^{2+}\mathrm{PL}]}{[\mathrm{PL}]_f[\mathrm{Mn}^{2+}]_f} \quad (5)$$

where $[Mn^{2+}PL]$ is the concentration of bound Mn^{2+}, eq 4 and 5 may be combined to give

$$\Delta \equiv 1/T_2 - 1/T_{2_0} = \frac{K_{eff}[\mathrm{Mn}^{2+}]_0}{(1 + K_{eff}[\mathrm{PL}]_0)}\frac{q}{(T_{2m} + \tau)} \quad (6a)$$

In cases where the effective equilibrium constant is not known, an upper limit of Δ is given by

$$\Delta \equiv 1/T_2 - 1/T_{2_0} \leqslant \frac{[\mathrm{Mn}^{2+}]_0 q}{[\mathrm{PL}]_0(T_{2m} + \tau)} \quad (6b)$$

Employing eq 6a and 6b and the data from Figure 2, some parameters describing the interaction between Mn^{2+} and sonicated PC and sonicated PG dispersions can be estimated. A comparison of Δ for the outside phosphorus and choline $N^+(CH_3)_3$ proton resonances of sonicated PC reveals that their ratio is 8. Assuming that K_{eff} is the same for both nuclei and that the denominator in eq 6a is dominated by T_{2m}, we obtain that the ratio of the T_{2m} of the ^{31}P resonance to that of the choline $N^+(CH_3)_3$ proton resonance is 8. Since T_{2m} depends on the distance between the Mn^{2+} ion and the nuclei, the correlation times of the complex and the magnitude of the hyperfine interaction (Solomon, 1955; Bloembergen, 1951), additional experiments are required before we can employ the ratio of the T_{2m} of the phosphorus and the choline $N^+(CH_3)_3$ proton resonances for calculating the ratio of their distances from the Mn^{2+} ion. However, a reasonable interpretation of this ratio is that in the Mn^{2+}–PC complex, the ion is located closer to the phosphorus than to the choline $N^+(CH_3)_3$ protons. This interpretation is in accord with the data shown in Figure 5, which suggests that when Eu^{3+} is bound to the outside PC it is located closer to the phosphorus than to the choline $N^+(CH_3)_3$ protons (McConnell and Robertson, 1958; Eaton and Phillips, 1965). Our conclusion regarding the location of the bound Mn^{2+} and Eu^{3+} agrees with studies of PC monolayers (Shah and Schulman, 1967) which indicate that Ca^{2+} bound to PC monolayers is in closer proximity to the phosphorus than to the choline $N^+(CH_3)_3$ protons.

The determinations of $T_{2m} + \tau$ for the phosphorus and the choline $N^+(CH_3)_3$ protons is not yet possible since the equilibrium constant and q are not known. However, assuming $q = 4$, we calculated (using eq 6b) an upper limit of $T_{2m} + \tau$ for the phosphorus and the choline $N^+(CH_3)_3$ resonances to be 1.2×10^{-5} and 9.6×10^{-5} sec, respectively. Since the outside phosphorus resonance of sonicated PG was broadened beyond detection by the lowest Mn^{2+} concentration employed, only a lower limit for Δ can be obtained for PG. Thus applying eq 6b, we calculated an upper limit of $T_{2m} + \tau$ to be 7×10^{-6} sec. It is of interest to note that the outside phosphorus resonance was broadened beyond detection by less than one ion per vesicle. This observation raises the possibility that a given ion broadens most of the "outside" resonances of a given PG vesicle before it diffuses to another vesicle. However, several diffusion processes can determine τ:diffusion of Mn^{2+} from the complex into the bulk solvent, diffusion of Mn^{2+} to other PG molecules, and the exchange of PG molecules into and out of the Mn^{2+} complex; therefore we cannot as yet conclude which process is rate limiting. In any case, 7×10^{-6} sec is an upper limit for the fastest of these processes.

From eq 6a we see that the difference in the value of several parameters could explain the observation that Mn^{2+} broadens the outside PG phosphorus resonance more than that of PC. This difference might arise from stronger binding of Mn^{2+} to the negatively charged PG than to the zwitterionic PC, a smaller T_{2m} for the PG ^{31}P resonance than for the PC ^{31}P resonance, or both.

Appendix 2

The Mn^{2+} broadening of the resonances of the nuclei present on the "outside" of cosonicated PG:PC vesicles can be used to elucidate the spatial arrangement of PG and PC within the outer monolayer. We assume that Mn^{2+} is in equilibrium between being free in the bulk solution and being bound to the vesicles, that Mn^{2+} can form complexes, designated $Mn^{2+}PL_{ij}$, with i molecules of PC and j molecules of PG, that all the complexes have the same total number of phospholipid molecules q (*i.e.*, $q = i + j$), and that in a given complex the same nuclei of the bound PC and PG each have a single transverse relaxation time which we designate respectively as $T_{2PC_{ij}}$, and $T_{2PG_{ij}}$. We further assume that the nature of the $Mn^{2+}PL_{ij}$ complex is independent of the neighbors of the PG and PC molecules in the complex (this assumption may not always be valid, see last section of this appendix). Since in our experiments the total Mn^{2+} concentration, $[Mn^{2+}]_0$, is much smaller than the total concentration of the outside PC, $[PC]_0$, and of the outside PG, $[PG]_0$, we may use the Swift and Connick equation (1962). Equation 7 is the expression for the Mn^{2+} broadening of the "outside" PC resonances.

$$\Delta_{PC} \equiv 1/T_2 - 1/T_{2_0} = \sum_{\substack{i=0 \\ i+j=q}}^{q} \frac{[\mathrm{Mn}^{2+}\mathrm{PL}_{ij}]}{[\mathrm{PC}]_0}\frac{i}{(T_{2\mathrm{PC}ij} + \tau_{ij})} \quad (7)$$

where τ_{ij} is the lifetime of each complex. A similar equation can be written for the PG resonances. If we assume that the free Mn^{2+} is in equilibrium between the bulk solution and the $Mn^{2+}PL_{ij}$ complexes, we may define apparent equilibrium constants K_{ij}

$$K_{ij} = \frac{[\mathrm{Mn}^{2+}\mathrm{PL}_{ij}]}{[\mathrm{PL}_{ij}]_f[\mathrm{Mn}^{2+}]_f} \quad (8)$$

where $[Mn^{2+}]_f$ is the concentration of Mn^{2+} in the bulk solution and $[PL_{ij}]_f$ is the concentration of sites where Mn^{2+} binds to i PC and to j PG molecules. Since $[PL_{ij}]_f$ is a function of the total phospholipid concentration, we may define (eq 9) a proportionality constant, α_{ij}, between these quantities.

$$[PL_{ij}]_f = \alpha_{ij}([PC]_0 + [PG]_0) \quad (9)$$

The proportionality constants may be affected by the ratio $[PG]_0/[PC]_0$, and may also be a function of the Mn^{2+} concentration. This coefficient is of great significance, since knowing its value for all the possible PL_{ij} is equivalent to knowing the spatial arrangement of PG and PC within the surface. Equations 7–9 may be combined to give eq 10. A similar expression may be written for the PG resonances.

$$\Delta_{PC} \equiv 1/T_2 - 1/T_{2_0} = \sum_{\substack{i=0 \\ i+j=q}}^{q} \frac{iK_{ij}\alpha_{ij}([PC]_0 + [PG]_0)}{\left\{1 + ([PC]_0 + [PG]_0)\left(\sum_{\substack{i=0 \\ i+j=q}}^{q} K_{ij}\alpha_{ij}\right)\right\}} \frac{1}{(T_{2PC_{ij}} + \tau_{ij})} \quad (10)$$

If we assume that the phospholipids are arranged in large patches which contain significantly more molecules in their center than on their boundaries, then eq 10 can be simplified to

$$\Delta_{PC} \equiv \frac{1}{T_2} - \frac{1}{T_{2_0}} = \frac{K_{PC}[Mn^{2+}]_0 q}{(1 + K_{PC}[PC]_0 + K_{PG}[PG]_0)} \frac{1}{(T_{2PC} + \tau)} \quad (11)$$

where K_{PC} and K_{PG} are the equilibrium constants of Mn^{2+} with PC and PG as defined in Appendix 1. A comparison of eq 11 of this appendix and eq 6a of Appendix 1 reveals that under this assumption the outside resonances of both PC and PG should be broadened less when these molecules are in the cosonicated vesicles than when they are in the respective pure vesicles. However, using the data shown in Figures 2 and 5, we find that the outside PC ^{31}P resonance is broadened more when PC is in the cosonicated vesicles than when it is in the PC vesicles, whereas for PG the opposite holds. If we assume more elaborate arrangements, then eq 10 rather than eq 11 has to be applied to our experimental results. Since eq 10 contains more variables, the analysis becomes very complicated. However, with reasonable estimates of the relative magnitude of the unknown parameters our data suggest that PG and PC are not arranged in small patches even if the boundaries are included. Thus we may conclude that within the nmr experiment time scale PG and PC are not segregated in the outer monolayer of the cosonicated vesicles; a more precise statement about the arrangement of these molecules in the bilayer cannot as yet be given.

We made two simplifying assumptions in the derivation of eq 10: that the Mn^{2+} forms only one type of complex with i PC molecules and j PG molecules, where always $i + j = q$, and that the nature of the complex is independent of the nearest neighbors of the phospholipids in the complex. The first assumption is not a necessary requirement for showing that our data are inconsistent with a spatial arrangement where PG and PC are segregated into large patches. The second assumption may not always be justified, but if most of the PG and PC molecules were segregated, then most of the Mn^{2+} complexes would have been in either a PG or a PC environment and therefore this assumption would not have affected eq 10.

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Application of Laser Self-Beat Spectroscopic Technique to the Study of Solutions of Human Plasma Low-Density Lipoproteins†

R. W. DeBlois,* E. E. Uzgiris, S. K. Devi, and Antonio M. Gotto, Jr.*

ABSTRACT: The technique of self-beat or homodyne spectroscopy has been applied to the determination of the translational diffusion constant of human plasma low-density lipoproteins (LDL). Both power spectrum and autocorrelation methods give equivalent results, but with conventional equipment the power spectrum measurement takes on the order of hours while autocorrelation is accomplished in minutes. The effects of concentration of LDL, scattering angle of the light pH of the solution and buffer concentration were investigated. The diffusion constant obtained, $D_{25,w} = 2.14 \pm 0.09 \times 10^{-7}$ cm²/sec, was in good general accord with conventional measures. The equivalent spherical diameter, obtained from the Stokes–Einstein relationship, 229 ± 10 Å was within a broad band of values given by other techniques.

The translational diffusion constant, D, has been classically used in biochemistry for the determination of the molecular weight of proteins in conjunction with sedimentation data and for the calculation of frictional ratios of proteins. While sedimentation equilibrium methods have become popular for molecular weight determinations of smaller proteins, these methods cannot generally be applied to large particles such as the plasma lipoproteins. The measurement of D has traditionally been done by experiments in which the spreading of the boundary is measured as the protein being studied diffuses into a solvent layer (Longsworth, 1945). The most accurate measurements of D have been by the spreading boundary technique (Gosting, 1956). In recent years, the measurement of the D of macromolecules has been greatly simplified by a technique involving the analysis of laser light scattered from a suspension of the molecules in question (Chu, 1970; Cummins and Swinney, 1970). The Brownian motion of scattering macromolecules causes the scattered beam to be slightly broadened in frequency. This effect has been used by Dubin *et al.* (1967), who obtained physical information about biological macromolecules from the power spectrum of scattered light. While recording the power spectrum requires on the order of hours with conventional equipment, we have been able to obtain comparable results in minutes with the adjunct of autocorrelation. It is the purpose of this communication to describe how laser homodyne spectroscopy may be conveniently applied to the determination of the translational diffusion constant of human plasma low-density liporpoteins (LDL).

Theory

For a selected scattering angle the frequency spectrum of photocurrent is measured from a photodetector exposed to the scattered light. The square of the amplitude of the output of the photodetector at a given frequency, within a constant frequency band, is proportional to the spectral power, $S(f)$, of the photocurrent. For a monodisperse system of scatterers, the component of the power spectrum that arises from the random motions of scatterers can be shown to have a Lorentzian form, *i.e.*

$$S(f) \propto 1/(1 + f^2/f_0^2) \quad (1)$$

This spectral power is in addition to the shot noise of the detector and electrical noise of associated circuits. The Lorentzian band is centered around zero frequency. The half-width frequency at $^1/_2$ the maximum power, f_0, of Lorentzian spectrum is proportional to the translational diffusion constant of the scattering molecules. Specifically, if monochromatic light of wavelength λ is scattered through an angle θ in a medium of index of refraction n by particles of diffusion constant D, then

$$f_0 = 16\pi Dn^2 \sin^2(\theta/2)/\lambda^2 \quad (2)$$

A second method, complementary to that of measuring the power spectrum of the fluctuations in intensity of scattered light, is that of measuring the autocorrelation function of the

† From the General Electric Research and Development Center, Schenectady, New York 12301 (R. W. D. and E. E. U), The National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland, and The Department of Medicine, Baylor College of Medicine, The Methodist Hospital, Houston, Texas 77025 (S. K. D. and A. M. G.). *Received March 24, 1973*. Presented in preliminary form at the 16th Annual Meeting of the Biophysical Society, Toronto, Canada, Feb 24–27, 1972 (*Biophys. Soc. Abstr., 12*, 140a). This work was supported in part by Contract NIH-NHLI-71-2156 from the National Heart and Lung Institute, by HEW Grant HL 14194, and by a grant from the John A. Hartford Foundation, Inc.