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Structure of Macromolecular Aggregates. II. Construction of Model Membranes from Phospholipids and Polypeptides*

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ABSTRACT: The interactions of aqueous phospholipid dispersions (liposomes) with a variety of water-soluble polypeptides were investigated as models for the molecular interactions occurring in biological membranes. Circular dichroism, optical rotatory dispersion, electron microscopy, nuclear magnetic resonance, and stopped-flow techniques were utilized. Major changes in polypeptide conformation, the physical state of the lipid fatty acid chains, the morphology of the liposomes, and the permeability of the liposomes were found to occur. The data suggest that the phosphatidyl-L-serine–poly-L-lysine complex (pH 7.0) is stabilized by both electrostatic and hydrophobic interactions. The poly-L-lysine goes from a random coil to an α helix when it interacts with phosphatidyl-L-serine. A dramatic rearrangement of the liposomes into large aggregates of membrane-like vesicles is seen under the electron microscope. This is accompanied by a relative immobilization of the lipid fatty acid chains.

The liposomes behave as semipermeable membranes unless a molar residue excess of poly-L-lysine is present in the lipid–peptide complex. Phosphatidyl-L-serine also increases the helix content of poly-L-ornithine (pH 10.0) and poly Glu^{12}-$Lys^{28}Ala^{30}$ (pH 5.0); the interaction with poly-L-ornithine produces the same vesicular aggregates under the electron microscope as poly-L-lysine. A 1:1 molar mixture of phosphatidyl-L-serine and phosphatidylcholine is much less than half as effective at increasing the helicity of poly-L-lysine as phosphatidyl-L-serine alone. Polypeptides with net negative charges were not found to interact with phosphatidylcholine or phosphatidyl-L-serine.

Biological membranes vary greatly in composition (Korn, 1969; Rothfield and Finkelstein, 1968), but consist mainly of protein (20–70%) and lipid, and in some cases small amounts of carbohydrate and nucleic acid. The protein includes a variety of enzymes, "structural" protein, and some which seems to serve both roles (Racker and Bruni, 1968); the lipid is predominantly phospholipid. A great deal of work has been done to characterize the physical–chemical behavior of phospholipids (Bangham, 1968; Dervichian, 1964), but relatively little is known about the nature of lipid–protein interactions and their role in stabilizing membrane structure.

Aqueous phospholipid dispersions (liposomes) consisting of closed, concentric bimolecular layers separated by aqueous channels are attractive model membranes. Inasmuch as they consist of bimolecular phospholipid layers, they are quite similar to the structure proposed for biological membranes in the "unit" membrane model (Robertson, 1966) in which a phospholipid bilayer is coated on both sides with protein. Liposomes of variable but known composition are easily prepared. In fact, the properties of liposomes made from a variety of natural and synthetic phospholipids have been found to mimic the behavior of native membranes in many respects, including permeability, thermotropic liquid crystalline phase transitions, and sensitivity to hormones, antibodies, detergents, and drugs (Bangham, 1968; Steim, 1968; Sessa and Weissmann, 1968). Synthetic, water-soluble polypeptides have been extensively studied as model proteins (Fasman, 1967), and in general their physical properties are much easier to interpret in terms of structure than those of proteins.

In a previous study (Hammes and Schullery, 1968), quite drastic conformational changes were found to occur as a result of polypeptide–polypeptide interactions. In the present work, circular dichroism, optical rotatory dispersion, electron microscopy, nuclear magnetic resonance, and stopped-flow kinetics were employed to study the interactions of liposomes

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with a variety of water-soluble polypeptides. These simple model systems should provide a basis for the interpretation of physical measurements on membrane-like suspensions, and may provide some insight into the molecular interactions that are of importance in biological membranes. The interactions of phospholipids with polypeptides were found to produce major changes in polypeptide conformation, the physical state of the lipid fatty acid chains, the morphology of the liposomes, and in some cases the permeability of the liposomes.

Experimental Section

Materials. Phosphatidyl-L-serine, isolated from bovine brain, was purchased from General Biochemicals (lot 87078) and used without further purification. Thin-layer chromatography on silica gel plates using a $CHCl_3$–CH_3OH–CH_3COOH–H_2O (25:15:4:2) solvent phase and I_2 vapor for developing indicated minor phosphatidylethanolamine contamination. Similar circular dichroism results were obtained using PhdSer[1] from Pierce Chem. Co. (lot 03109-4). PhdCh was isolated from chicken egg yolks as described elsewhere (Hammes and Roberts, 1970).

Poly-Lys, poly-L-Orn, poly-L-Glu, poly $Glu^{42}Lys^{28}Ala^{30}$, and poly $Glu^{60}Lys^{40}$ were purchased from Pilot Chemical Co. and were reported to have the following molecular weights: poly-Lys (lot L-77), 100,000; poly-L-Orn (lot 0-19), 90,000; poly-L-Glu (lot G-80), 86,000; poly GluLysAla (lot M-21D), 30,000; poly GluLys (lot C-35), 90,000. Poly-Lys, poly-L-Orn, and poly-L-Glu were dialyzed and stock solutions prepared as previously described (Hammes and Schullery, 1968). The copolymers were dialyzed against NaF, followed by H_2O, filtered to remove some insoluble material, and then lyophilized. Stock solutions in H_2O were prepared and their concentrations were determined by micro-Kjeldahl analysis. All other chemicals used were commercial analytic reagent grade.

Experimental measurements were made at ~25°, except for the nuclear magnetic resonance studies, which were done at ~28°.

Circular Dichroism. A 1% stock dispersion of PhdSer in H_2O was prepared by stirring the dry PhdSer and H_2O for a few hours. The dispersion was then sonicated in an ice bath, under N_2, using a Branson Sonifier with a microtip; a nearly clear solution was obtained after 6-min sonication. This solution was stored at 4° under N_2 and remained suitable for use for several weeks. Sample solutions were prepared by adding an aliquot of the stock PhdSer dispersion in a disposable lambda pipet to a few milliliters of the polypeptide solution followed by rapid swirling. The concentration of polypeptide was generally $1–2 \times 10^{-4}$ M. The pH of the polypeptide solutions was checked and adjusted as necessary before and after the addition of PhdSer. Stock dispersions of PhdCh and 1:1 PhdCh–PhdSer were prepared and used in the same manner as the PhdSer dispersion. The solutions were buffered as described in the figure legends.

Circular dichroism and optical rotatory dispersion measurements were performed on a Cary 60 spectropolarimeter with circular dichroic attachment using a 1-cm path-length cell. In spite of the turbidity of the solutions, the spectra were reproducible to within a few millidegrees, and the noise level was not any larger than normally encountered except at very short wavelengths. The mean residue rotation, $[m]$, and mean residue ellipticity, $[\theta]$, were calculated in the usual manner (Yang, 1967; Scanu and Hirz, 1968).

Osmotic Permeability. The water permeability of liposomes is amenable to study by monitoring the initial rate of volume change upon exposure to an osmotic pressure gradient (Bangham *et al.*, 1967; de Gier *et al.*, 1968). The volume change is proportional to $1/A$, where the absorbance, A, is entirely due to light scattering, and can be conveniently monitored spectrophotometrically.

Solutions were prepared as previously described. The solutions for this work were not sonicated, and sucrose was used as the osmotically active species. Equal volumes of PhdSer or PhdSer–poly-L-lysine complex were mixed with aqueous sucrose in a stopped-flow apparatus (Aminco-Morrow or Durrum-Gibson) in about 5 msec. The change in transmittance was then monitored at 375 nm on a storage oscilloscope, where the trace could be photographed with an attached Polaroid camera. To determine the initial rate of volume change, the slope of the early part of the trace was calculated (in % transmission/sec) and then converted into $d(1/A)/dt$, which is proportional to the rate of volume change. The time range for most of the swelling and shrinking ranged from 0.2 to 3 sec.

Nuclear Magnetic Resonance. The PhdSer dispersions and poly-L-lysine solutions used in the nuclear magnetic resonance work were prepared as above except they were made up in D_2O (99.75%). The sonicated solutions were sonicated for 15 min in an ice bath under N_2 using a microtip at a power output of 55 W. Sonication made the PhdSer dispersion much clearer. At these concentrations the PhdSer–poly-L-lysine mixtures were extremely turbid and visibly particulate. Sonication of the PhdSer–poly-L-lysine complex had only a small visible effect, and that was to eliminate the larger particles: no noticeable clearing of the dispersion occurred.

The nuclear magnetic resonance spectra were taken on a Varian Associates HA-100-IL nuclear magnetic resonance spectrometer connected to a Varian C 1024 time-averaging computer. A coaxial insert containing tetramethylsilane was used to provide a lock signal. Because prevention of large-scale precipitation required use of relatively low concentrations, the computer was used to average the spectra of repetitive scans. Resonance peaks were identified by comparison with published spectra (Chapman and Morrison, 1966; Joubert *et al.*, 1969).

Results

Circular Dichroism. The circular dichroism of PhdSer–poly-L-lysine complexes was studied in the far-ultraviolet region, where the rotatory strength of the peptide chromophore has been shown to be a sensitive function of the backbone conformation (Greenfield and Fasman, 1969). The PhdSer alone had no detectable optical activity under conditions employed in this work. The circular dichroism spectra in Figure 1 of poly-L-lysine at pH 7.0 and 11.6 are characteristic of the random coil and α-helical polypeptide conformations, respectively. Also shown are representative spectra

[1] Abbreviations used are: PhdSer, phosphatidyl-L-serine; PhdCh, phosphatidylcholine.

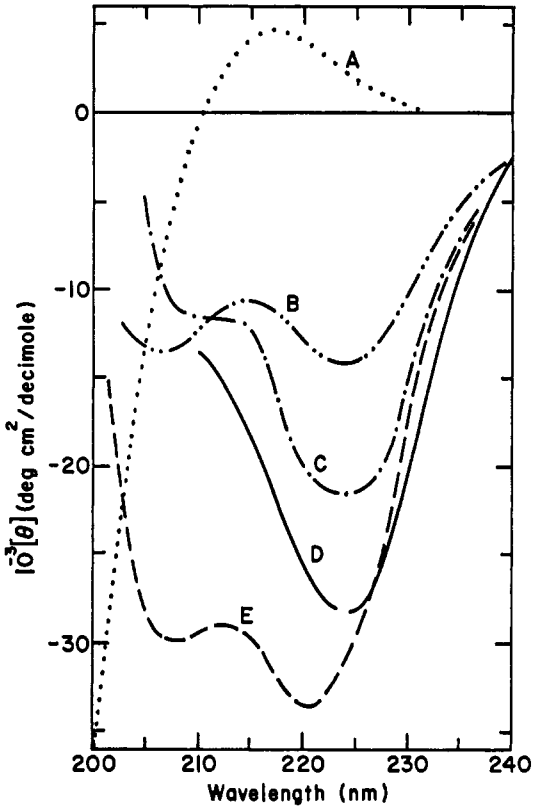

FIGURE 1: Plot of the mean residue ellipticity, $[\theta]$, *vs.* wavelength for poly-L-lysine and PhdSer–poly-L-lysine complexes: A–D pH 7.0, 0.01 M Tris-HCl; (A) poly-L-lysine; (B) 0.5:1 mole residue ratio of PhdSer–poly-L-lysine; (C) 0.75:1 mole residue ratio of PhdSer–poly-L-lysine; (D) 1:1 mole residue ratio of PhdSer–poly-L-lysine; (E) poly-L-lysine, pH 11.6, 0.01 M NaH_2BO_3.

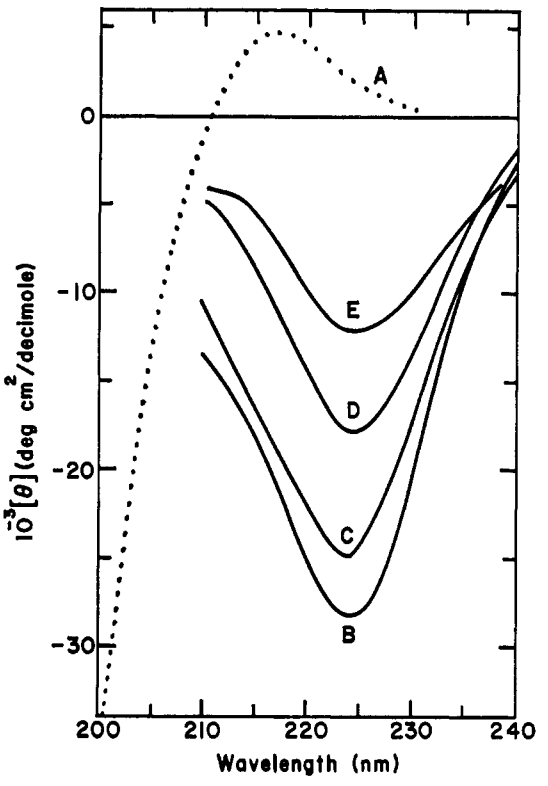

FIGURE 2: Plot of the mean residue ellipticity, $[\theta]$, *vs.* wavelength showing the salt effect on the 1:1 mole residue ratio PhdSer–poly-L-lysine complex, pH 7.0, 0.01 M Tris-HCl: (A) poly-L-lysine alone; (B) PhdSer–poly-L-lysine; (C) PhdSer–poly-L-lysine, 0.10 M NaCl; (D) PhdSer–poly-L-lysine, 0.25 M NaCl; (E) PhdSer–poly-L-lysine, 1.0 M NaCl.

of the PhdSer–poly-L-lysine complex at varying mole residue ratios. The trough at 222 nm was found to red shift to ~225 nm and to smoothly increase in amplitude as the mole residue ratio of PhdSer:poly-L-lysine was varied from 1:20 to 1:1. Except for the red shift, the circular dichroism spectra at the higher mole residue ratios of PhdSer:poly-L-lysine have the characteristics associated with an α-helical structure, and this is presumed to be the structure formed. However, the possibility exists that other ordered structures are formed. The characteristic 208-nm α-helix trough was smaller relative to the 222-nm trough than is usually the case; however it has been our experience that this is a common phenomenon in highly aggregated helical systems (Hammes and Schullery, 1968; also unpublished results). For the more turbid solutions, the high absorbance due to light scattering prevented investigation of the 208-nm trough. The solutions grew more turbid as the charge equivalence point was approached, and in the worst cases were only stable for a few hours before precipitation occurred. The maximal increase in helix content corresponded to ~85% helix if the value of $[\theta]_{222}$ for the helix in solution is assumed to be valid in this system. This assumption is highly questionable (Hammes and Schullery, 1968) and probably underestimates the amount of helix (Ji and Urry, 1969).

Figure 2 illustrates the effect of increasing ionic strength upon the circular dichroism of the 1:1 PhdSer–poly-L-lysine complex. A slight decrease in the magnitude of $[\theta]_{225}$ occurred upon going to 0.1 M NaCl and larger decreases upon going to 0.25 and 1.0 M NaCl. Accompanying the decrease in $[\theta]_{225}$ was a greatly increased turbidity, presumably due to salting the aggregates out of solution. Under these conditions it cannot be said with certainty what portion of the decrease in signal is due to a decrease in helix content of the poly-L-lysine and what portion is due to merely a lowering of the effective concentration by formation of aggregates so large as to be impenetrable by the light.

A 1:1 molar mixture of PhdSer and PhdCh was much less effective (Figure 3) in increasing the helix content of poly-L-lysine than was PhdSer alone. Only a small increase occurred, even at pH 9.55 where poly-L-lysine is beginning to become uncharged (Ciferri *et al.*, 1968).

No significant difference was seen between the effectiveness of PhdSer and PhdSer–cholesterol mixtures (with up to 37.5 mole % of cholesterol) in increasing the helix content of poly-L-lysine (pH 7). The turbidity of dispersions of both lipid alone and the lipid–polypeptide complexes increased as the mole per cent of cholesterol increased.

Optical rotatory dispersion spectra of PhdSer–poly-L-lysine complexes were rather ambiguous under conditions of very high turbidity which still yielded reasonable circular dichroism spectra. The characteristic α-helix optical rotatory dispersion trough at 233 nm was replaced by a large positive rotation ($[m]_{233} \approx 25{,}000$ deg cm^2/dmole), which decreased in amplitude, but remained positive to beyond 450 nm. This behavior is probably an instrumental artifact. The

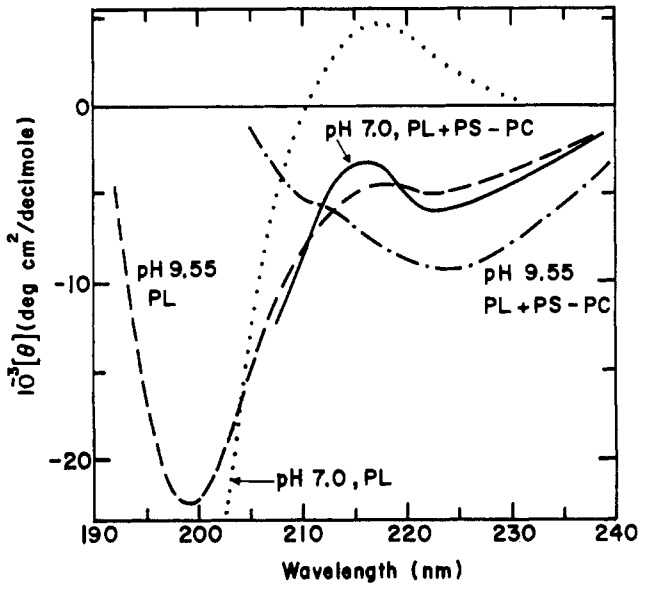

FIGURE 3: Plot of the mean residue ellipticity, $[\theta]$, *vs.* wavelength for poly-L-lysine and poly-L-lysine + 1:1 molar ratio of PhdCh-PhdSer at pH 7.0, 0.01 M Tris-HCl and at pH 9.55, 0.01 M NaH_2BO_3. The mole residue ratio of poly-L-lysine:phospholipid was 1:1.

optical rotatory dispersion for a less turbid solution exhibited the 233-mμ trough, but the crossover point was red shifted from 223 to ~228 nm.

An attempt was made to study the PhdSer–poly-L-lysine complex in the presence of the negative stain used for the electron microscopy, 1% ammonium molybdate (pH 7.0). (The most popular negative stain, sodium phosphotungstate could not be used for these purposes since it precipitated poly-L-lysine from solution.) The high absorbance of 1% ammonium molybdate below 275 nm prevented a direct examination of the circular dichroism or optical rotatory dispersion in the region of the peptide chromophore; however the long-wavelength optical rotatory dispersion tail could still be observed and was utilized to monitor the coil–helix

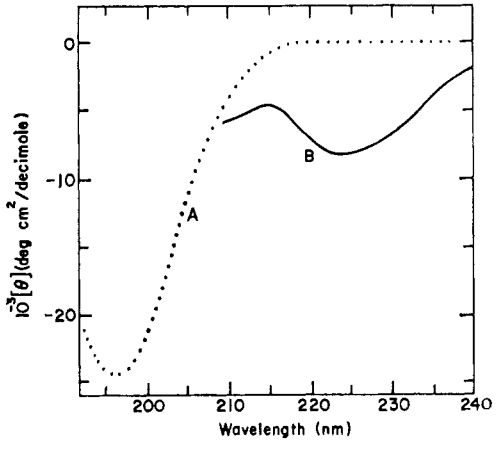

FIGURE 4: Plot of the mean residue ellipticity, $[\theta]$, *vs.* wavelength, pH 10.0, 0.01 M NaH_2BO_3: (A) poly-L-ornithine; (B) 1:1 mole residue ratio of poly-L-ornithine:PhdSer.

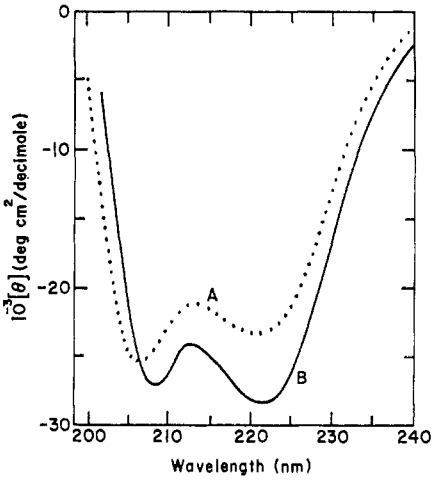

FIGURE 5: Plot of the mean residue ellipticity, $[\theta]$, *vs.* wavelength, pH 6.00, 0.01 M $Na_2H_2P_2O_7$: (A) poly $Glu^{42}Lys^{28}Ala^{30}$; (B) 1:1 average mole residue ratio of $Glu^{42}Lys^{28}Ala^{30}$:PhdSer.

transition through the b_0 parameter (Yang, 1967). Poly-L-lysine in 1% ammonium molybdate was found to undergo a normal coil–helix transition upon pH titration, the b_0 parameter being −520 deg cm²/dmole at pH 12.5. Unfortunately, the b_0 parameter proved to be unreliable for the highly aggregated, PhdSer–poly-L-lysine complex systems. The b_0 parameter did not vary smoothly as the PhdSer:poly-L-lysine ratio was adjusted so as to increase the helicity of poly-L-lysine. Instead, b_0 ranged from zero to positive (250 deg cm²/dmole), with a high degree of experimental scatter in the plots. However, this behavior was the same in the presence or absence of the 1% ammonium molybdate, which suggests the stain does not markedly alter the macromolecular conformation.

PhdSer slightly increased the helix content of poly-L-ornithine (Figure 4) when added at pH 10.0, where the poly-L-ornithine coil has started to become less stable due to the partial deprotonation of the side-chain amino groups (Chaudhuri and Yang, 1968). The increase in $[\theta]_{222}$ corresponded to about a 20% increase in helix content, again using the $[\theta]_{222}$ value for a helix in solution as a reference point. At pH 7 extensive turbidity developed indicating some interaction but there was no detectable circular dichroism signal; this could be due either to destruction of the order that gives rise to the "random" coil spectrum, or to the very extensive aggregation. At pH 11, where poly-L-ornithine is less highly charged, no visible evidence of interaction and no change in the circular dichroism upon addition of PhdSer occurred.

The interaction of the random copolymers poly $Glu^{42}Lys^{28}Ala^{30}$ and poly $Glu^{60}Lys^{40}$ with PhdSer was investigated. Both polymers exhibited an isoelectric precipitation around pH 5.5 in the absence of added salt, and were found to be in a mostly helical form below that pH. At pH 5.0, upon addition of PhdSer, poly GluLysAla underwent about a 12% increase in helix content (Figure 5). The complex was not visibly turbid, and no red shift occurred upon complex formation. Poly GluLys also exhibited a comparable increase in $[\theta]_{222}$ upon addition of PhdSer at pH 5.0, but the meaning of this effect was quite ambiguous since solutions both with and without PhdSer were visibly turbid. Neither copoly-

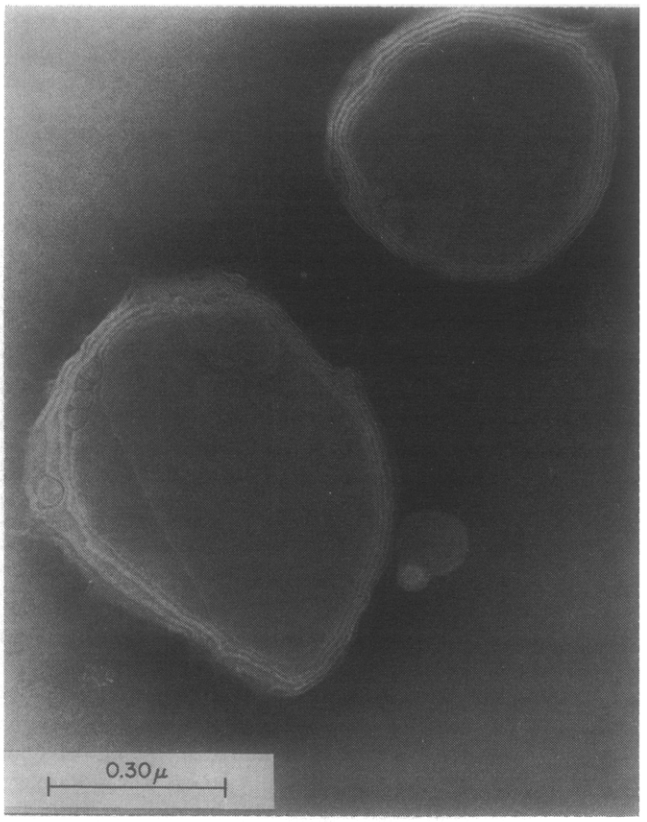

FIGURE 6: Electron micrograph of PhdSer dispersed in 1% ammonium molybdate, pH 7.0.

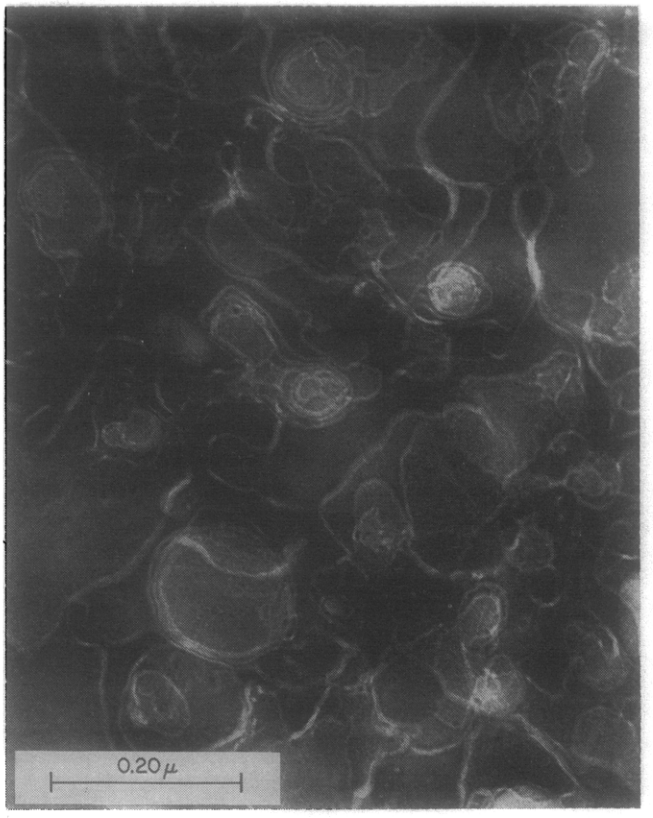

FIGURE 7: Electron micrograph of 0.77:1 mole residue ratio PhdSer–poly-L-lysine complex in 1% ammonium molybdate, pH 7.0.

mers showed any evidence of interaction with PhdSer at pH values where they had a net negative charge.

The interaction of PhdCh liposomes with all of the above polypeptides and poly-L-glutamic acid was investigated by circular dichroism over a wide range of pH. Very small and nonreproducible increases in helix content were observed with poly-L-Lys, but no evidence of interaction with any of the other polypeptides was found.

Electron Microscopy. When viewed in the electron microscope after negative staining (Valentine and Horne, 1962) with 1 or 2% sodium phosphotungstate or ammonium molybdate, PhdSer liposomes exhibited the commonly reported (Bangham, 1963) concentric bimolecular lipid layer appearance as shown in Figure 6. Great heterogeneity existed with respect to particle size; diameters ranged from about 250 Å to 1 μ. Often smaller liposomes were seen trapped within larger ones. Generally the liposomes seemed to have a lipid center, although frequently they consisted of only a few bilayers with an aqueous center. Of the two negative stains, ammonium molybdate gave the more satisfactory results, and all of the work reported here was done with it.

When a 1% ammonium molybdate solution of poly-L-lysine was mixed with a PhdSer dispersion in 1% ammonium molybdate in proportions yielding a mole residue excess of either poly-L-lysine or PhdSer, a dramatic rearrangement of the liposome structure occurred. Large aggregates of membrane-like vesicular structures are formed as shown in Figure 7. Interspersed throughout the aggregates are small liposomes apparently in varying states of partial disruption. The poly-L-lysine itself was not visible in the micrographs. Also, no fine structure in the bilayers indicative of pores or subunits could be resolved. Due to the apparent variation in bilayer thickness seen in any one micrograph, no attempt was made to compare this quantity in the presence and absence of the polypeptide. Identical results were obtained when poly-L-ornithine was substituted for poly-L-lysine (Figure 8). No change in the PhdSer liposome structure was observed upon addition of the copolymers poly Glu^{42}-$Lys^{28}Ala^{30}$ at pH 5, 6, or 7, or poly $Glu^{60}Lys^{40}$ at pH 6 or 7. The work at lower pH values was complicated by the tendency of ammonium molybdate to crystallize upon drying on the grid.

Osmotic Permeability. The initial rates of volume change of PhdSer liposomes and PhdSer–poly-L-lysine complexes when exposed to an osmotic pressure gradient were studied under a variety of conditions. The initial shrinking rate data for water dispersions of PhdSer and PhdSer–poly-L-lysine (7.7:1 mole residue ratio) show a linear dependence on the osmotic pressure gradient when mixed with equal volumes of sucrose solutions (Figure 9). The linear dependence is expected for an ideal semipermeable membrane (Stein, 1967), *i.e.*, one completely impermeable to solute. A PhdSer dispersion prepared 11 days before the experiment showed considerable deviation from linearity at the higher osmotic pressure differences. This deviation is consistent with an increased permeability to sucrose, which might be due to partial oxidation of the PhdSer. The PhdSer–poly-L-lysine complex seemed to shrink about half as fast (*i.e.*, be half as permeable to water) as the PhdSer alone; however in

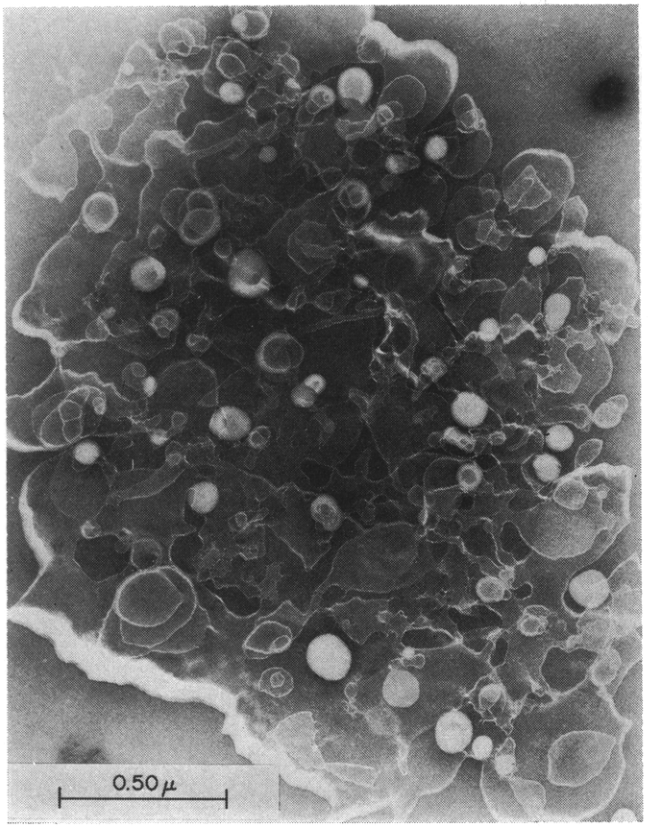

FIGURE 8: Electron micrograph of 0.77:1 mole residue ratio PhdSer–poly-L-ornithine complex in 1% ammonium molybdate, pH 7.0.

light of the extensive aggregation in the complex, this difference cannot be attributed to a real change of bilayer permeability with any certainty.

Both shrinking and swelling experiments were done on PhdSer and PhdSer–poly-L-lysine complexes (7.7:1) prepared in 0.1 M sucrose. Again, as shown in Figure 10, linear dependence of the rate on osmotic pressure gradient was found. Within the experimental error, no difference was found in the apparent rates for the PhdSer and PhdSer–poly-L-lysine complex.

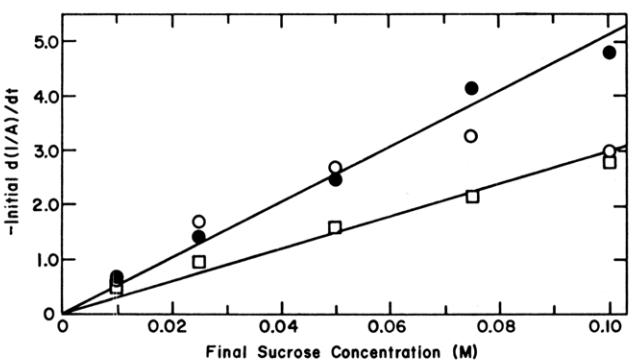

FIGURE 9: Initial shrinking rate, $d(1/A)/dt$ *vs.* final sucrose concentration for PhdSer and PhdSer–poly-L-lysine dispersions prepared in H_2O and mixed with aqueous sucrose solutions: (●) fresh PhdSer dispersion; (○) 11-day-old PhdSer dispersion; (□) 7.7:1 mole residue ratio PhdSer–poly-L-lysine dispersion.

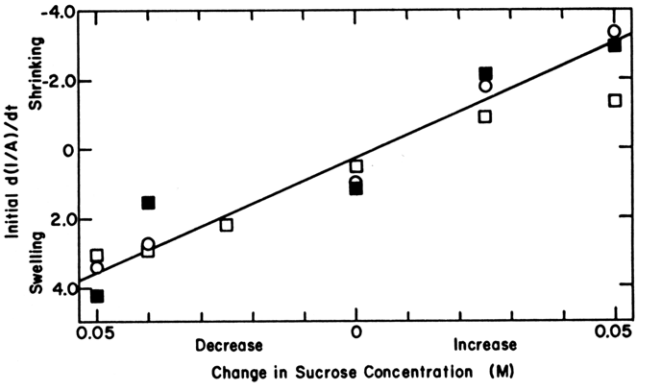

FIGURE 10: Initial shrinking and swelling rates, $d(1/A)dt$, *vs.* change in sucrose concentration for PhdSer and PhdSer–poly-L-lysine dispersions prepared in 0.1 M sucrose and mixed with higher and lower concentrations of sucrose solution: (○) PhdSer dispersion; (■, □) (separate experiments), 7.7:1 mole residue ratio PhdSer–poly-L-lysine dispersion.

The permeability of PhdSer–poly-L-lysine complexes prepared in water and then mixed with an equal volume of 0.2 M sucrose was investigated for a variety of PhdSer–poly-L-lysine ratios. Complexes with a PhdSer:poly-L-lysine mole residue ratio of 2.8 to 19 exhibited shrinking behavior similar to the work reported thus far. A complex with PhdSer:poly-L-lysine = 1:1 could not be used for these experiments since it precipitated too rapidly from solution. A complex with PhdSer:poly-L-lysine = 0.57:1 did not behave as a closed, semipermeable membrane. Upon mixing a water dispersion of this complex with either 0.2 M sucrose or water, small and variable decreases in the absorbance of the solution were seen, whereas the other systems investigated under these "shrinking" conditions exhibited an increase in absorbance. No distinguishable difference was visible by electron microscopy between the PhdSer–poly-L-lysine complexes with a molar excess of PhdSer or poly-L-lysine.

The concentration dependence of the shrinkage rate of water dispersions of PhdSer upon being mixed with 0.2 M sucrose was determined over the concentration range 0.005–0.05 wt %. A decrease in the shrinkage rate of about one order of magnitude occurred in going from low to high concentrations, a constant value being reached at about 0.015%. Over the same concentration range Beer's law is observed quite precisely. The change in permeability may be related to an aggregation phenomenon.

Nuclear Magnetic Resonance. Nuclear magnetic resonance spectra were taken of unsonicated and sonicated PhdSer, poly-L-lysine, and PhdSer–poly-L-lysine solutions. The results are summarized in Figure 11. Unsonicated PhdSer exhibited a very broad resonance due to the fatty acid hydrogens with an upfield shoulder due to the terminal CH_3. The poly-L-lysine spectrum consisted of two peaks, both from side-chain protons. The ϵ-CH_2 resonance was at τ 6.8. The upfield resonance (τ 8.3) consisted of a larger peak due to the γ-CH_2 and δ-CH_2 protons with an upfield shoulder due to the β-CH_2 protons. The α-CH resonance could not be resolved among the computer-accumulated spinning side bands of the HOD peak. In the PhdSer–poly-L-lysine complex (1:3), the PhdSer resonance was apparently completely broadened, presumably due to the fatty acid hydro-

carbon chains becoming immobilized. The poly-L-lysine resonances were slightly reduced in intensity but not chemically shifted. The absence of a chemical shift is evidence that the ϵ-NH_3^+ has remained protonated (Joubert *et al.*, 1969).

As reported by others (Chapman *et al.*, 1968; Steim, 1968), sonication considerably sharpened the methylene proton resonance of the phospholipid; however in the mixture of [sonicated PhdSer]–poly-L-lysine (1:3), the PhdSer resonance was again completely broadened. Sonication of this PhdSer–poly-L-lysine mixture slightly increased the intensity of the poly-L-lysine peaks but did not visibly sharpen the PhdSer peak. A [sonicated PhdSer]–poly-L-lysine mixture with a molar excess of PhdSer (3.3:1) showed a PhdSer resonance that was broadened compared to sonicated PhdSer alone but was readily detectable; this mixture was approximately as turbid as the 1:3 PhdSer–poly-L-lysine mixture. The poly-L-lysine concentration was too low in this solution for detection of its nuclear magnetic resonance spectrum.

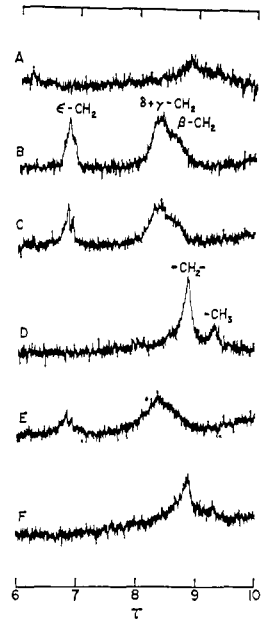

FIGURE 11: 100-MHz nuclear magnetic resonance spectra of PhdSer, poly-L-lysine, and PhdSer–poly-L-lysine complexes in D_2O. Spectra D, E, and F were recorded at twice the sensitivity as A, B, and C. (A) Unsonicated 2% PhdSer, 21 scans; (B) 1.5% poly-L-lysine, 21 scans; (C) 2% PhdSer–1.5% poly-L-lysine (1:3 mole residue ratio of PhdSer:poly-L-lysine), 21 scans; (D) sonicated 2% PhdSer, 6 scans; (E) sonicated mixture of [sonicated 2% PhdSer]–1.5% poly-L-lysine (1:3 mole residue ratio of PhdSer:poly-L-lysine), 6 scans; (F) [sonicated 2% PhdSer]–0.15% poly-L-lysine (3.3:1 mole residue ratio of PhdSer:poly-L-lysine), 6 scans.

Discussion

The results presented here show that major changes in molecular structure can occur when polypeptides and phospholipid liposomes interact. Circular dichroism and optical rotatory dispersion changes indicate alteration of the polypeptide conformation upon addition of liposomes. For the systems studied, electrostatic attraction is a fundamental requirement for interaction of peptide and lipid; circular dichroism changes or turbidity development was never found when the polypeptide had a net negative charge. The majority of the work reported here was done using PhdSer liposomes; PhdSer has approximately one net negative charge per molecule over the pH range 5–9.5 (Papahadjopoulos, 1968). Very small and nonreproducible effects are seen when PhdCh (zero net charge) liposomes are used. When 1:1 PhdSer–PhdCh liposomes are mixed with poly-L-lysine, the circular dichroism change is considerably less than half of that produced by PhdSer alone. From these results, a specific requirement for the PhdSer polar moiety cannot be ruled out. When the electrostatic requirement is met, the interaction is generally helix stabilizing. This is most dramatically seen with the PhdSer–poly-L-lysine mixtures at pH 7.0 in which poly-L-lysine is converted from random coil into essentially all helix; poly-L-lysine does not normally begin to become helical until about pH 10 (Fasman, 1967). This tendency for helix stabilization is not too surprising since charge repulsion is a primary force in destabilizing the helix, although, as was reported earlier poly-L-lysine can become stabilized in either the β-pleated sheet or the α helix upon aggregation with polyacids (Hammes and Schullery, 1968). Poly-L-ornithine is known to form a helix much less readily than poly-L-lysine (Chaudhuri and Yang, 1968), but a definite increase in helicity is seen upon addition of PhdSer liposomes at pH 10.0. Poly $Glu^{42}Lys^{28}$-Ala^{30}, which is predominantly helical at pH values below its isoelectric point (Schullery, 1970), undergoes about a 12% increase in helix content upon addition of PhdSer at pH 5.0. This is the maximum helicity obtainable by pH titration (Schullery, 1970).

Although electrostatic binding seems to be the predominant factor in peptide–lipid complex formation, hydrophobic bonding is probably also contributing to the stability of the complex, since salt concentrations as high as 1 M NaCl failed to completely disrupt the PhdSer–poly-L-lysine complex. An ionic strength of 0.1 M is effective in disrupting the ionic complexes formed by mixed phospholipids and cytochrome *c* (Green and Fleischer, 1964), whereas 0.1 M NaCl had only a minor effect on the PhdSer–poly-L-lysine complex. The absence of any effect of cholesterol on the PhdSer–poly-L-lysine interaction suggests that the hydrophobic core of the PhdSer bilayers is not extensively involved in the interaction, inasmuch as cholesterol is known to be located near the fatty acid chains in mixed cholesterol–phospholipid liquid crystals (Lecuyer and Dervichian, 1969). Several instances have been reported in the literature where both ionic and hydrophobic effects seemed to be important in the interaction of phospholipids and proteins (Green and Fleischer, 1964; Gulik-Krzywicki *et al.*, 1969; Braun and Radin, 1969; Sweet and Zull, 1969; Sessa *et al.*, 1969). The decrease in the magnitude of $[\theta]_{225}$ which occurs upon increasing the ionic strength of the PhdSer–poly-L-lysine complex could be due either to partial disruption of the complex or to the greatly increased turbidity caused by salting the complexes out of solution.

The optical rotatory parameters have now been reported for a variety of biological membranes (Steim, 1968), and the common features of these data are red-shifted spectra corresponding to a mixture of α-helix and random coil conformations, and reduced intensity of the helix peaks as compared with the solubilized membrane protein. Both intensity reduction and red shift are reported to occur upon aggregation of

isolated structural protein (Steim and Fleischer, 1967), so the lipid may or may not be involved in the effect observed with intact membranes. This work shows that a red shift and an increase in the intensity of the characteristic helix peaks can occur upon aggregation of phospholipid and polypeptides. In one system, PhdSer–poly $Glu^{42}Lys^{28}Ala^{30}$, an increase in helix content is observed without any red shift; perhaps of significance is that this was the only complex which was not visibly turbid. Other workers have reported an increase in helix content upon addition of phospholipids to apo-high-density serum lipoprotein (Scanu and Hirz, 1968), and an increase in β structure upon addition of phospholipids to apo-low-density serum lipoprotein (Gotto *et al.*, 1968). No changes in optical rotatory spectra were found when cytochrome c was complexed with mixed beef heart phospholipids (Ulmer *et al.*, 1965). Clearly, generalizations cannot yet be made predicting the effect of added phospholipids on protein conformation, but the results reported here indicate that at least for polypeptides quite dramatic effects are possible.

Electron microscopy using negative staining showed that PhdSer liposomes are converted into aggregates of membrane-like vesicular structures upon interaction with poly-L-lysine or poly-L-ornithine at pH 7. This result is rather similar to those reported upon mixing various phospholipids with some natural proteins (Revel and Ito, 1967; Stoeckenius, 1966; Papahadjopoulos and Miller, 1967). Although partially disrupted liposomes were also present in the aggregates, apparently the more membrane-like structure is favored in the presence of these polycations. This effect is not seen when poly $Glu^{60}Lys^{40}$ (pH 6, or 7) or poly $Glu^{42}Lys^{28}Ala^{30}$ (pH 5, 6, or 7) are substituted for poly-L-lysine or poly-L-ornithine. The structure of the vesicular aggregates probably consists of single PhdSer bilayers with a polypeptide coating on the surfaces. Extensive penetration of the polypeptide chain into the bilayer probably does not occur since the basic bilayer appearance is unchanged. However, the salt effects previously discussed indicate hydrophobic interactions are of some importance, and therefore some penetration must occur.

The electron micrographs of the PhdSer–poly-L-lysine complex are the same regardless of whether the PhdSer or poly-L-lysine is in excess, but the osmotic permeability experiments indicate quite different behavior for these two cases. When PhdSer is in excess, the complex behaves as an ideal semipermeable membrane, *i.e.*, it is impermeable to sucrose; this is the same behavior seen with PhdSer liposomes. When poly-L-lysine is in excess, osmotic behavior of the complex is not observed, presumably due either to the existence of pores large enough to allow rapid penetration of both solute and solvent, or to the formation of membrane-like sheets rather than closed vesicles. The similarity of the electron micrographs of both complexes suggests the first alternative is more probable.

The nuclear magnetic resonance data offer some indication of a molecular structural change which may be related to the observed permeability behavior. The data suggest a gradual freezing-in of the PhdSer fatty acid chains upon addition of increasing amounts of poly-L-lysine. The exact meaning of nuclear magnetic resonance studies on membranes and phospholipids is subject to considerable uncertainty due to the correlation of phospholipid nuclear magnetic resonance peak height with sonication time (Steim, 1968; Chapman *et al.*, 1968). The origin of this dependence is not understood since the only other known effect of sonication is to favor formation of smaller liposomes (Chapman *et al.*, 1968). Less efficient tight packing of lipid molecules due to the smaller radius of curvature of the bilayers in sonicated liposomes has been suggested as a possible explanation (Steim, 1968). Our result showing a relatively sharp PhdSer spectrum for the 3.3:1 PhdSer–poly-L-lysine complex is of special interest in that this solution was extremely turbid, obviously consisting of much larger particles than in a simple PhdSer dispersion. This indicates that the mere fact of having large particles does not necessarily broaden the phospholipid methylene resonance peak.

The behavior of phosphatidylethanolamine dispersions may be relevant for understanding the results reported here. Phosphatidylethanolamine has been shown to form typical lipid bilayers upon dispersion in water and examination under the electron microscope (Papahadjopoulos and Miller, 1967); however permeability studies indicate that the bilayers are not closed as is found for other phospholipids (Papahadjopoulos and Watkins, 1967). Also phosphatidylethanolamine undergoes the liquid crystal phase transition to melted fatty acid groups at a higher temperature than other phospholipids with the same fatty acid composition (Steim, 1968), indicating that something peculiar about the polar moiety results in the formation of bilayers in which the fatty acids are relatively immobile. The characteristics of phosphatidylethanolamine are similar to those of the PhdSer–poly-L-lysine complex containing excess poly-L-lysine.

A simple model consistent with the data for the interaction of simple, water-soluble polypeptides and phospholipids involves the following points. (1) An important requirement for interaction of lipid and peptide is electrostatic attraction. (2) The interaction is partially stabilized by hydrophobic bonding, but this probably does not involve extensive penetration of the bilayer core by the polypeptide. (3) The interaction favors the formation of membrane-like vesicles consisting of a single phospholipid bilayer coated with the polypeptide, which can be a helix or a random coil. (4) The helix content of the polypeptide tends to be increased upon complexing with phospholipid. (5) The mobility of the fatty acid groups on the phospholipid is reduced as a result of the interaction. This is probably due to an induced tighter packing of the polar moieties in their crystalline array. (6) In the presence of excess polypeptide the vesicles develop pores or channels which destroy the osmotic behavior found in complexes with excess PhdSer.

This model membrane system is now quite well characterized in terms of structure and physical properties. Whether or not polypeptide–phospholipid vesicles are appropriate models for biological membranes is an open question, but in any event the results obtained should be of some help in interpreting physical measurements on biological membranes.

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