# A LAMELLAR COMPLEX OF LECITHIN AND POLY-L-TYROSINE

G. GIANNONI, F. J. PADDEN, JR., *and* R. J. ROE

*From the Department of Chemistry, New York University, New York 10013, and Bell Telephone Laboratories, Murray Hill, New Jersey 07974. Dr. Giannoni's present address is SNAM Progetti, Laboratorio Studi e Ricerche di Base, Monterotondo, Roma, Italy.*

ABSTRACT Complexes of poly-L-tyrosine (PT) with dipalmitoyllecithin, synthetic, (DPL) and with egg lecithin (EL) have been obtained by precipitation from methanol-water solutions. Chemical analysis indicates that both lecithins bind PT up to a limiting ratio of about 4 tyrosine residues/lecithin molecule. DPL-PT complexes have a lamellar structure closely resembling lecithin itself. In fact, DPL and DPL-PT lamellae have very nearly the same thickness as precipitated from methanol-water, although their swelling behavior on resuspension in pure water is different. The complexes crystallize in the form of hexagonal platelets, some monolayers and some with terraced spiral growths, with a thickness of 50–55 A. In X-ray and electron diffraction they yield sharp reflections at 4.14 A which are characteristic of hexagonal packing of phospholipid paraffinic chains. The order-disorder transition temperature of this crystalline lattice, determined by differential scanning calorimetry, is somewhat higher in the complex than in pure DPL. Physical models consistent with these observations are discussed.

## INTRODUCTION

An important question underlying the structure of cell membranes is the way in which polypeptides and phospholipids interact. Yet, because of the great chemical complexity of natural membranes, interactions between these molecules are poorly understood. A few model systems involving the binding of a purified protein to a phospholipid have been studied by various techniques (1). Even in such instances, it is extremely difficult to approach the degree of structural resolution required to understand the spatial relationships of binding. Proteins have many chemical functions, and different kinds of amino acid side chains are likely to interact in different ways with a given phospholipid. As a result of the lack of basic information, there is a disparity of views on the nature of protein-lipid associations in these model systems, and hence in cell membranes themselves. It seems desirable, therefore, to turn to the study of much simpler model systems. A simple approach, by which different kinds of interactions can also be distinguished and separately studied, is to investigate complex formation between phospholipids and polypeptides of a simple

composition, e.g., homopolypeptides. It has been shown, for example, that phosphatidyl-L-serine, in aqueous solution at appropriate pH, will interact with poly-L-lysine and poly-L-ornithine to form a complex stabilized partly by hydrophobic bonding, but principally by electrostatic attractions (2). We wish to describe a well defined lamellar complex of lecithin and PT, formed in the absence of dominant polar bonding, and to report some structural investigations which are relevant to this line of inquiry.

## MATERIALS AND METHODS

PT of low molecular weight, which is soluble in methanol and methanol-water mixtures, was purchased from Miles Laboratories, Inc. (Elkhart, Ind.). Two samples, with molecular weights of 5000 and 11,300 as determined by the supplier, were used with similar results, although some quantitative differences in binding behavior will be noted below. Dipalmitoyllecithin (1,2-dihexadecanoyl-*sn*-glycero-3-phosphorylcholine, synthetic) (DPL) was purchased from Nutritional Biochemical Corporation (Cleveland, Ohio) and from Calbiochem (Los Angeles, Calif.). Both samples were chromatographically homogeneous on thin-layer silica G plates, using as developer chloroform-methanol-water (65:25:4 by volume) and iodine vapor as a stain (3).

Chromatographically purified EL was purchased from Pierce Chemical Co. (Rockford, Ill.) in sealed ampuls as a concentrated solution in heptane. Working in a dry box flushed continuously with nitrogen, this solution was evaporated to dryness at room temperature, and methanol was added to the solid residue to make a 2% solution which was distributed into well stoppered flasks and stored at −20°C until used. This material was homogeneous in thin-layer chromatography except for a very small amount of an impurity with slightly higher $R_f$.

Quantitative determinations of lecithin were performed by phosphate analysis after Kjeldahl digestion (4). Determination of PT was done spectrophotometrically, using a molar extinction coefficient (on a residue weight basis) of $2.25 \times 10^3$ at 2940 A in 0.1 N NaOH (5). When lecithin is also present, dilute alkali is not a convenient solvent for spectrophotometry. Accordingly, ultraviolet spectra were measured in methanol, in which the molar extinction coefficient was found to be $1.60 \times 10^3$ at 2780 A. At this wavelength, and at concentrations which are of interest here, EL has very little absorption; therefore, neither lecithin interferes with the spectrophotometric determination of PT.

For X-ray diffraction studies suspensions of crystalline complexes in mother liquor were concentrated by centrifugation and sealed in liquid-tight sample holders. These had windows of mica or polystyrene film. A Guinier-DeWolff focusing camera was used to record on film diffraction patterns in the wide-angle region and also in the moderately low-angle region corresponding to spacings up to 32 A. A Kratky camera fitted with a wide entrance slit of 400 $\mu$ was used to record intensities in the angular region corresponding to spacings of 150–10 A. Slit desmearing, by a computer program according to Schmidt (6), was applied before measurement of peak positions and integrated intensities.

For transmission electron microscopy and electron diffraction, crystals were deposited from mother liquor onto carbon-coated specimen support grids, dried in air, and shadowed witn platinum-carbon at a distance:height ratio of 1:1.

Thermal measurements were made on a Perkin-Elmer differential scanning calorimeter, DSC-1 (Perkin-Elmer Corp., Norwalk, Conn.).

## RESULTS

### *Formation of PT-Lecithin Complexes*

1% solutions of PT and of EL in methanol both remain clear upon the addition of 0.25 volumes of water at room temperature; however, the same addition of water will produce an abundant precipitate in a mixture of the two solutions over a wide range of PT:EL molar ratios. Precipitates redissolve upon heating, and reform on cooling to room temperature. Under the phase-contrast optical microscope, they appear microcrystalline and sometimes distinctly lamellar. Chemical analyses to be reported below show that these precipitates consist of complexes of PT and EL. The method of precipitation outlined above seems to afford the highest degree of crystalline order, and was therefore adopted throughout this work.

If more water is added, a similar precipitation behavior is observed with PT or EL alone, but the appearance of these precipitates is quite different, especially with EL, from which suspensions of liposomes are obtained.

DPL also forms a complex with PT; however, because DPL is less soluble than EL in methanol-water mixtures, the formation of a complex cannot be so easily demonstrated by differential solubility at room temperature. Addition of water to precipitate the complex will also cause any excess DPL to separate as a pure lamellar phase, which can be recognized in the phase-contrast microscope by its sheetlike appearance. This complication can, however, be avoided. In the methanol-water mixtures used in this study (5:1 and 4:1 by volume) and at concentrations below 1%, DPL does not come out of solution as a pure solid down to 40°C, while it will form mixed precipitates with PT well above that temperature, as is shown in Table I. These precipitates can be separated from excess reactants in the mother liquor by

TABLE I

CRYSTALLIZATION TEMPERATURES OF DPL-PT MIXTURES IN METHANOL-WATER, 5:1 (v/v)

| DPL | PT* | $R_{mixt}$‡ | Crystallization range§ |
|---|---|---|---|
| *mg/ml* | *mg/ml* | | *°C* |
| 8.3 | — | — | 37.5–36.5 (38.5–38.0) |
| 4.1 | — | — | 35–34 |
| 4.1 | 4.1 | 4.7 | 67–49 |
| 4.1 | 2.05 | 2.35 | 57–49 |
| 4.1 | 0.82 | 0.94 | 56–48 (61–53) |
| 4.1 | 0.41 | 0.47 | 56–45 |
| — | 4.1 | — | No precipitation down to 20°C |

* PT mol wt 5000.

‡ Molar ratio of PT (on a residue basis) to lecithin in the mixture.

§ The first temperature marks the onset of opalescence, the second is approximately the point at which the precipitate coagulates. Data in parentheses refer to mixtures in methanol-water, 4:1 (v/v).

spinning in a clinical centrifuge thermostated at 40°C, and quickly removing the supernatant by suction. By this method DPL-PT complexes were routinely prepared for further study.

*Chemical Characterization of Complexes*

After conditions were found which allowed the isolation of complexes from unreacted components, analysis of the precipitates was carried out in order to investigate the stoichiometry of binding. In a series of precipitations the molar ratio PT: lecithin in the mixture ($R_{mixt}$) was varied over a 10-fold range, other conditions being kept constant. The PT:lecithin molar ratio in the corresponding precipitates ($R_{ppt}$) was determined by chemical analysis. Results for DPL and EL are compared in Table II. It is clear that both lecithins can bind PT up to a limiting ratio of about 4 tyrosine residues/phosphorus.

The mixture methanol-water-chloroform (5:2:1) is a solvent for both DPL and PT (mol wt 5000). If solutions are mixed at 20°C, precipitation of complexes takes place over a wide range of PT:DPL ratios, although yields are much lower than in the methanol-water system. One analysis was performed to determine the composition of a complex precipitated from methanol-water-chloroform at room temperature. The concentration of DPL in the original mixture was 4 mg/ml and $R_{mixt}$ was 0.45. $R_{ppt}$ was found to be 3.8, in good agreement with previous results.

Binding behavior in methanol-water shows a moderate dependence on the molecular weight of the polyaminoacid. Somewhat more extensive binding is observed with PT of lower molecular weight. This relationship, however, was not explored for any length, especially because PT of higher degree of polymerization (e.g., several hundreds) is not soluble in methanol.

Most of the following studies were carried out using polymer of molecular weight 5000.

TABLE II

ANALYSIS OF MIXED LECITHIN-PT PRECIPITATES

| $R_{mixt}$ | $R_{ppt}$ | |
|---|---|---|
| | DPL | EL |
| 0.9 | 2.3 (3.0) | 1.8 |
| 2.3 | 2.5 (3.4) | 2.3 |
| 4.7 | 2.8 (3.9) | 2.7 |
| 9 | 3.2 | 3.2 |

Solvent is methanol-water, 4:1 (v/v).
Total concentration of lecithin in all mixtures is 2.65 mg/ml.
Data in parentheses are for PT of mol wt 5000; all other data are for PT of mol wt 11,300.

*Ether Extraction of DPL-PT Complexes*

Precipitates of DPL obtained by cooling from solutions in methanol-water are easily dissolved by brief shaking with ethyl ether, while polytyrosine is not. In a test of their capability to withstand ether extraction, we found that precipitates of DPL-PT complex do not lose much lecithin by this treatment. Samples (5–10 mg) of precipitates obtained as in Table II were extracted twice by shaking for 20–30 sec with 3 ml of ether and analyzed. Results were as follows:

| $R_{mixt}$ | $R_{ppt}$ (unextracted) | $R_{ppt}$ (extracted) |
|---|---|---|
| 2.3 | 3.3 | 3.9 |
| 4.7 | 3.8 | 4.7 |

In both cases about 80% of the lecithin remained bound in the complex.

*Morphology of the Complexes*

DPL crystallizes from methanol-water mixtures in the form of wide, irregularly crumpled sheets. In the electron microscope, these vacuum-dried sheets were found by shadow casting to have a thickness of ~50 A. Electron diffraction patterns from these specimens consist of a set of six spots ($d$ = 4.15 A) corresponding to a hexagonal lattice. The sheets are therefore easily identified as phospholipid bilayers in which molecules, because of the chemical homogeneity of their fatty acid chains, can be ordered in a crystalline array. If PT is added to the system, precipitates show a morphological change. Broad sheets are replaced by lamellar aggregates and spherulites of varying complexity. The simplest crystals, i.e. well developed hexagonal monolayers and terraced spiral growths (Figs. 1 *a* and 1 *b*), are obtained from methanol-water (4:1) when $R_{mixt} \sim 3$. The thickness of these lamellae by shadow casting is 54 ± 5 A, and electron diffraction patterns are essentially identical to those of pure DPL. DPL-PT complexes, then, form a lamellar phase quite similar to lecithin. A puzzling feature of this structure already emerges from these observations. The binding of about 3.5 amino acid residues/lecithin molecule has taken place in such a way that (*a*) the thickness of the lamella remains virtually unchanged (see also X-ray evidence below), and (*b*) the hexagonal packing of the lecithin molecules is not appreciably disturbed.

EL separates from methanol-water systems not as crystalline monolayers, but rather in the well-known form of myelin figures or liposomes. In the presence of PT the morphology of precipitates is similar to that of DPL-PT complexes, although simple monolayers have not been obtained.

*X-Ray Diffraction*

Concentrated suspensions of DPL precipitated from methanol-water give X-ray powder diffraction pattern which are characteristic of powders of crystalline phos-

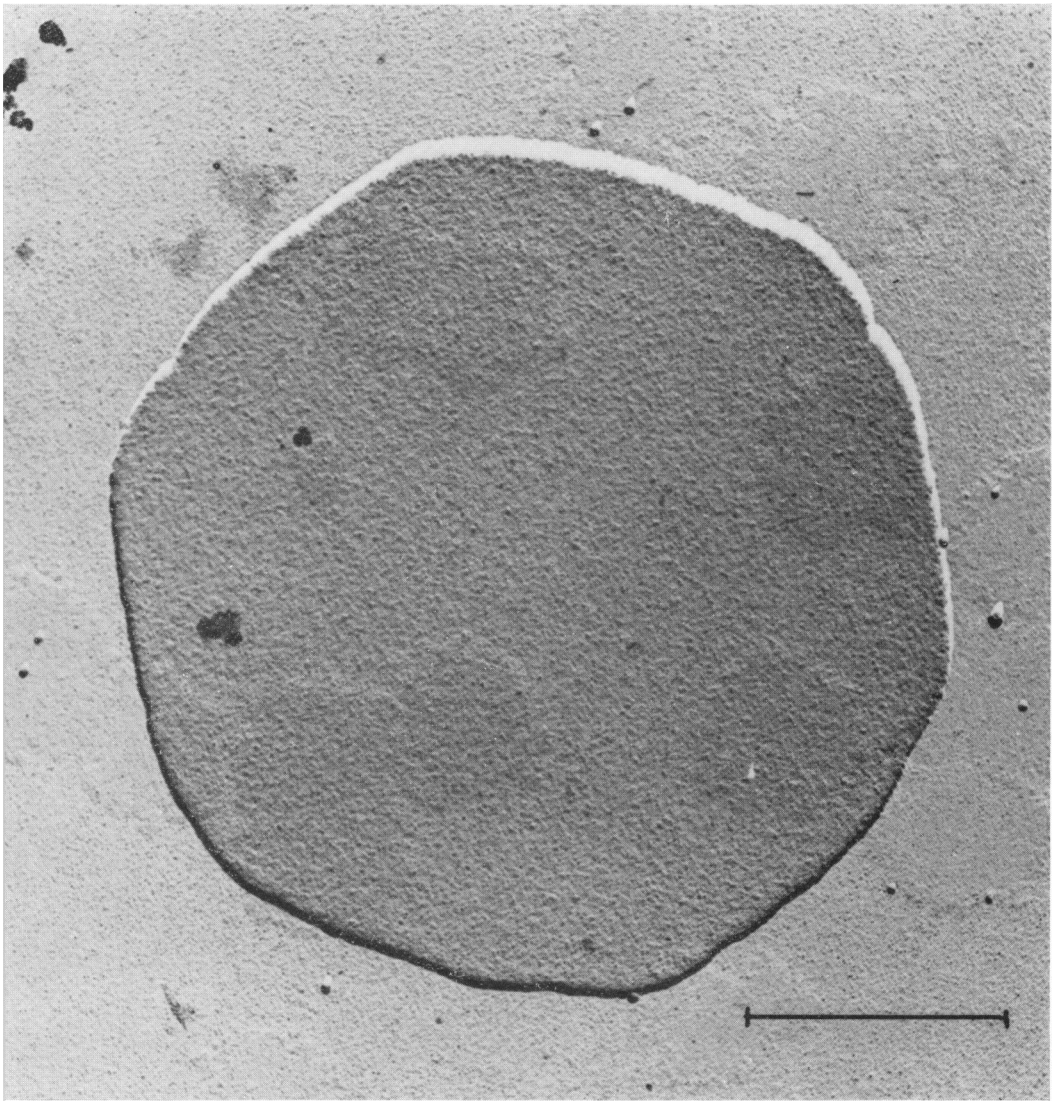

FIGURE 1 *a* Monolayer crystal of DPL-PT complex. $R_{mixt} \sim 3$. Bar represents 1 $\mu$. Shadowed with platinum-carbon at $\tan^{-1} 1/2$.

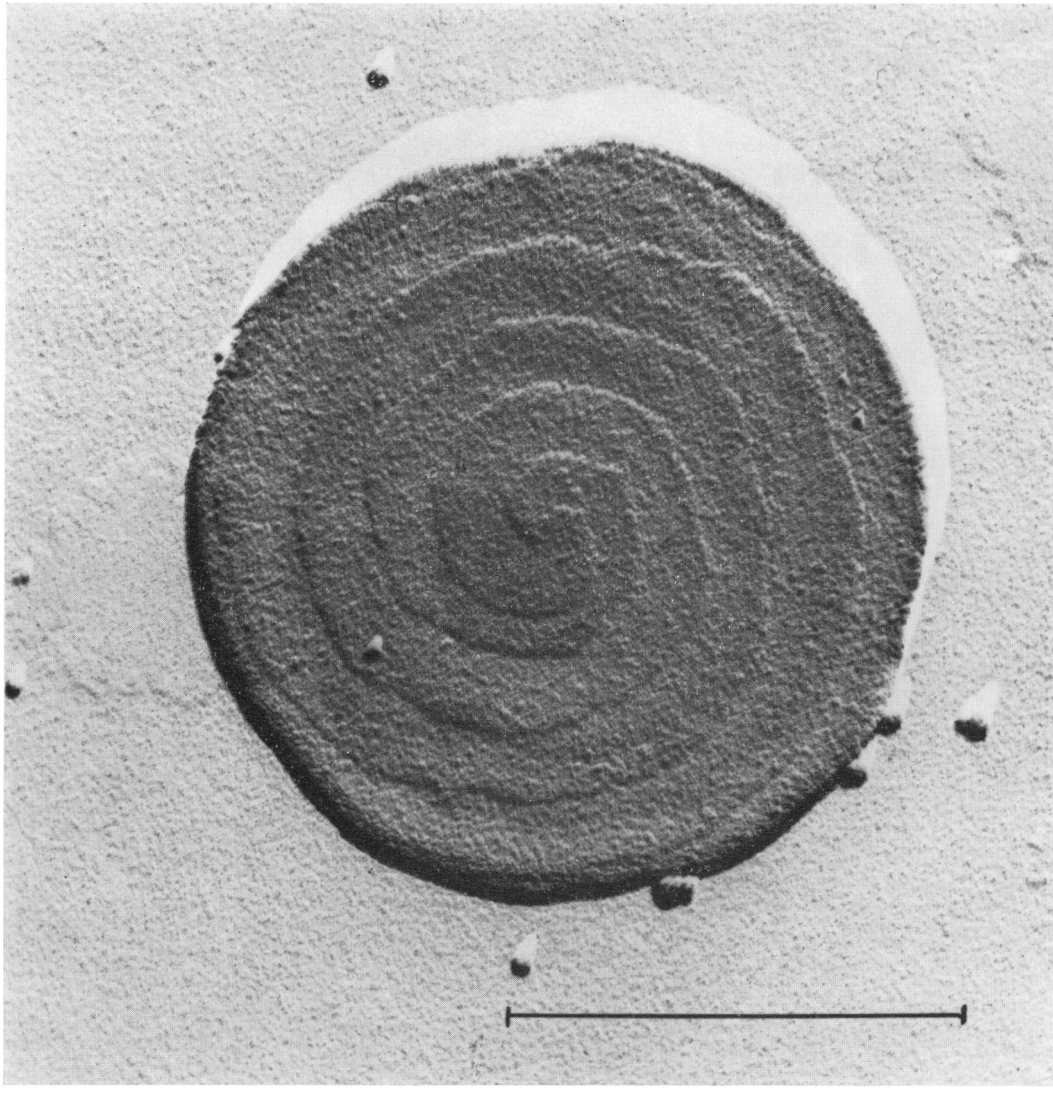

FIGURE 1 *b* Terraced multilayer crystal of DPL-PT complex. $R_{mixt} \sim 3$. Bar represents 1 $\mu$. Shadowed with platinum-carbon at $\tan^{-1} 1/2$.

pholipids (7). They consist of a strong, sharp reflection in the wide-angle region ($d$ = 4.14 A) which arises from the hexagonal packing of lecithin molecules within one bilayer, and of several orders of reflections corresponding to the "long spacing," $l$ (50–60 A), which arises from the stacking of many bilayers. The exact value of $l$ depends on the water content of the mother liquor. In mixtures with methanol:water ratios varying between 4:1 and 3:1, $l$ has a constant value of 51.5 A for DPL lamellae. This value is considerably smaller than the long spacing reported for dry crystalline powders of dipalmitoyllecithin (~60 A) (8), showing that in our suspensions the packing of DPL sheets is quite close. If DPL precipitated from methanol-water mixtures is spun down and resuspended in water, $l$ increases to 64.4 A.

Diffraction patterns from DPL-PT complexes exhibit the same reflections as those of pure DPL. The wide-angle reflection of 4.14 A remains as strong and sharp as before, indicating that complex formation does not disturb the crystalline packing of the paraffinic chains of DPL. In agreement with electron microscopic measurements, the long spacing $l$ is found to retain the value of 51.5 A observed for pure DPL in methanol-water mixtures. Comparisons of low-angle diffraction patterns from pure DPL and from DPL-PT complexes reveal one very conspicuous difference, namely that the second and third orders of the low-angle reflection, present in EL patterns, are invariably missing from DPL-PT patterns, although the fourth and fifth orders are observed. Patterns in which the second and third order of the long spacing are missing may be used, in fact, as a reliable indicator of the presence of the complex.

Resuspension of the DPL-PT complex in water does not significantly affect the

TABLE III

OBSERVED SPACINGS FOR LECITHINS AND FOR LECITHIN-POLYTYROSINE COMPLEXES

| | Long spacing | | Short spacing | |
|---|---|---|---|---|
| | In mother liquor* | In water | In mother liquor | In water |
| | *A* | *A* | *A* | *A* |
| DPL alone | 51.4 | 64.4 | 4.13 | 4.24 |
| EL alone‡ | | 64.4 | Very diffuse | |
| DPL-PT compex§ | 51.5 | 51.5 | 4.14 | 4.14 |
| EL-PT complex‖ | 53.6 | 60.3 | Very diffuse | |

*Mother liquor is methanol-water, 4:1, in each case.

‡ A methanol solution was evaporated under a stream of nitrogen, the residue was dried in vacuum over $P_2O_5$, then wetted with excess water for 18 hr before X-ray exposure.

§ $R_{mixt}$ = 2.3; PT mol wt, 5000.

‖ $R_{mixt}$ = 2.3; PT mol wt, 11,300.

4.14 A reflection, while it weakens considerably the rest of the pattern. The low-angle spacing $l$ is, however, undisturbed, in contrast to the behavior of DPL, thus indicating that DPL-PT complexes are less susceptible to solvation by water.

The diffraction pattern of pure EL is qualitatively similar to that of DPL, except that the 4.14 A reflection is replaced by an extremely broad band. This, a common diffraction feature in natural phospholipid mixtures when examined near room temperature, persists in the EL-PT complex which also has a lamellar structure. The EL-PT complex appears to be more susceptible to solvation by water than DPL-PT complexes. On transferring EL-PT from methanol-water (4:1) to pure water, $l$ increased from 53.6 to 60.3 A. It may be noted, however, that this EL-PT preparation had considerably lower $R_{ppt}$ than DPL-PT (See Table II).

### *Differential Scanning Calorimetry (DSC)*

For every phospholipid there exists a transition temperature $T_m$ at which the crystalline form is converted into a liquid-crystalline mesophase. In this conversion, which is an endothermic process, regular packing of the long-chain fatty acid residues is lost, to be replaced by a less ordered state. The precise value of $T_m$ depends on the state of solvation of the phospholipid, and it levels off to a minimum when a certain water content is reached (e.g., 30% for DPL) (8). Since this limiting value of $T_m$ is actually a measure of stability of crystalline packing of paraffinic chains, we measured the values of $T_m$ of DPL and DPL-PT complexes suspended in water for comparison. These samples were precipitated in the standard way from methanol-water (4:1), spun down, and resuspended in water by a brief sonication (80 kc, 80 w output). The materials were centrifuged from water suspensions and the pellets, with excess water added, were examined by DSC. Results shown in Table IV indicate that DPL-PT complexes have $T_m$ values a few degrees higher than DPL. Another difference is noted upon repetition of the heating and cooling cycles using the same specimen. On cycles subsequent to the first, DPL gives the same DSC diagram, while complexes yield their characteristic value of $T_m$ on the first heating only. In

TABLE IV

TRANSITION TEMPERATURES $T_m$ FOR DPL AND DPL-PT COMPLEXES

| | Transition temperature | |
|---|---|---|
| | First run | Subsequent runs |
| | °C | °C |
| DPL | 43.2 | 43.3 |
| DPL-PT: | | |
| $R_{ppt}$ = 3.9 | 46.7 | 41.6 |
| 3.4 | 46.9 | 43.2, 42.5, 42.2 |
| 3.0 | 48.2 | 44.0 |

all following cycles a markedly lower value is observed, which is close to that of pure DPL. Thus the initial "melting" of the complex in water is accompanied by some irreversible structural change.

## DISCUSSION

The isolation of lecithin-PT complexes containing up to about 4 amino acid residues/lecithin molecule provides a first clear instance of extensive binding between a phospholipid and a polypeptide in which ionic forces are apparently out of the question. Spontaneous formation of these lamellar structures, together with their high polypeptide:phospholipid ratio, also offers some hope for an approach to a model for cell membranes. An important finding is that the interaction between lecithin and polytyrosine follows a fairly well defined stoichiometry, suggesting that in each lecithin molecule there exist a definite number of binding sites to which polytyrosine residues are attached. This leads immediately to the two basic questions; where these binding sites are located on lecithin molecules, and what part of the polytyrosine monomeric unit is interacting with them. The mutual spatial arrangements of constituent molecules in the complex is no doubt determined to a large extent by the specific location of interacting sites. The evidence we have at hand is not sufficient yet to allow definitive conclusions on these questions; however, it severely restricts the choice of available models for the structure of the complexes, and deserves a more detailed discussion.

The basic requirements that have to be satisfied by any proposed structural model for the DPL-PT complex are: (*a*) the polypeptide must be accommodated (up to 4 amino acid residues/lecithin molecule) within a lamella of exactly the same thickness as that of pure lecithin, and (*b*) this must not involve extensive intermingling of polypeptide molecules with the paraffinic tails of the lecithin. The latter requirement is the consequence of our finding that the 4.14 A reflection from the DPL-PT complex is as sharp as from pure DPL. The slightly higher transition temperature $T_m$ observed for the complex as compared to that of pure DPL also suggests the same.

As possible arrangements of polypeptide and phospholipid components in the complex we consider the three models illustrated schematically in Fig. 2 *a-c*. In *a* and *b* the polypeptide interacts only with surface regions of the lecithin bilayers, very much as in the Davson-Danielli model of cell membrane structure, while in *c* the lecithin bilayer retains its original structure, but has inlaid into it patches or islands of polypeptide to create a mixed sheet with a mosaic distribution of components.

In model *a*, constancy of lamellar thickness is achieved through tilting of lecithin molecules in respect to the normal to the lamellar surfaces. The paraffinic tails of lecithin molecules are in almost the same environment as in pure lecithin and satisfy requirement *b*. The tilting of lecithin molecules, however, contradicts the evidence from electron diffraction. Three tyrosine residues will occupy roughly half of the

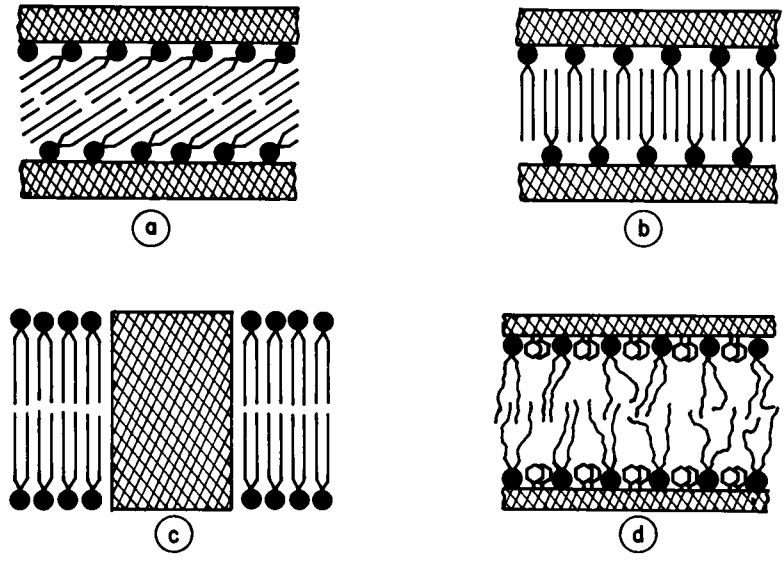


FIGURE 2 Schematic representations of three possible structural arrangements (*a–c*) of a DPL-PT complex having the same lamellar thickness as the lipid bilayer alone; *d* represents an EL-PT complex.

volume of a lecithin molecule, and therefore in the complex where $R_{ppt}$ is equal to 3 the lecithin molecules have to be tilted by about 45°. Selected-area electron diffraction from DPL-PT lamellae consistently yields six spots which are sometimes sharp, sometimes slightly arced, but never show any appreciable broadening or variation of intensity within a given pattern. Electron diffraction thus indicates that the paraffinic tails are oriented approximately normal to the lamellar planes, and certainly not tilted to the extent of 45° as demanded by model *a*.

In model *c* there is no difficulty in accounting for the constancy of the layer thickness. This model cannot, however, account easily for the fact that on suspension in water the complex does not show an increase in *I* while pure DPL does. The difficulty observed in extracting the lipid component from the complex with ether also points to the fact that lecithin molecules are shielded from solvents by their interaction with polypeptide molecules. It is possible that the mere existence of patches of polypeptide surrounding a column of lecithin molecules may afford some protection in the above sense; however, for this to be effective to the extent shown by the experimental evidence, the diameter of the lecithin column cannot be too great, and probably not more than several times the diameter of the paraffinic chains themselves. So finely subdivided a structure cannot, of course, satisfy the X-ray evidence of a sharp 4.14 A reflection and the DSC results.

In model *b* the nonpolar environment around the paraffinic chains is maintained when tails of opposing lecithin molecules become interdigitated. The X-ray and DSC results are therefore expected, since the packing and stability in the crystalline lattice

of paraffin chains should have remained essentially the same as in pure lecithin. The slight increase in $T_m$ in DPL-PT complexes can possibly be rationalized qualitatively by observing that on melting the paraffinic tails are confined to a smaller volume to move around, thus resulting in a smaller entropy of fusion. In fact, model *b* appears to satisfy all the experimental evidence we have, with the exception that it provides no explanation for the constancy of layer thickness *l* on complex formation. While appropriate structural devices for this purpose could be suggested in a speculative way, more definite conclusions must await further study, especially of X-ray diffraction intensities.

The weight of evidence certainly suggests that binding of PT involves the surface regions, not the interior of lecithin lamellae. This may no longer be true of interactions with polypeptides containing more hydrophobic side chains like leucine and valine, which are currently under study.

We are grateful to Mr. H. E. Bair for the DSC measurements, and to Dr. H. D. Keith for valuable discussions.

*Received for publication 14 June 1971.*

## REFERENCES

1. CHAPMAN, D., and R. B. LESLIE. 1970. Membranes of Mitochondria and Chloroplasts. American Chemical Society Monograph No. 165, New York. 91.
2. HAMMES, G. G., and S. E. SCHULLERY. 1970. *Biochemistry.* **9**:2555.
3. SINGLETON, W. S., M. S. GRAY, M. L. BROWN, and J. L. WHITE. 1965. *J. Amer. Oil Chem. Soc.* **42**:53.
4. JONES, A. S., W. A. LEE, and A. R. PEACOCKE. 1951. *J. Amer. Chem. Soc.* **133**:623.
5. FASMAN, G. D., E. BODENHEIMER, and C. LINDBLOW. 1964. *Biochemistry.* **3**:1665.
6. SCHMIDT, P. W. 1965. *Acta Crystallogr.* **19**:938.
7. FINEAN, J. B. 1953. *Biochim. Biophys. Acta.* **10**:371.
8. FINEAN, J. B., and P. F. MILLINGTON. 1955. *Trans. Faraday Soc.* **51**:1008.