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STRUCTURAL INVESTIGATIONS OF LIPID, POLYPEPTIDE AND PROTEIN MULTILAYERS

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SUMMARY

Langmuir–Blodgett multilayers of lipids, polypeptides and proteins have been examined by X-ray diffraction and infrared spectroscopic methods. The complex polymorphism exhibited by multilayers of glycerides and various phospholipids of different chain length mirror those shown in other three-dimensional structures and suggest that multilayers of lipids can be considered as oriented "crystals". Both the α and β types of hydrocarbon chain packing are adopted by different classes of lipids in multilayers.

Stable multilayers of the synthetic polypeptide poly-γ-benzyl-L-glutamate consist of α-helical rods stacked in an hexagonal array with a rod axis separation of 14.2 Å. Poly-γ-methyl-L-glutamate behaves similarly but little structural information could be derived from potentially non-helical or sheet-like structures formed by other homopolypeptides. The observation of a single, invariant diffraction line at 9.3 Å for multilayers of a number of water-soluble proteins is consistent with the occurrence of extensive structural reorganization (uncoiling, denaturation) at the air–water interface.

INTRODUCTION

In the 1930s, Blodgett and Langmuir[1] demonstrated that ordered arrays of surface-active molecules can be deposited from monolayers onto a solid surface (for a review see ref. 2). The imposition of such long-range order can be important in structural studies with biologically significant molecules and recently oriented multilayers of phosphatidylcholine (lecithin) formed on a solid support have been used to obtain additional molecular detail of the structure of phospholipid bilayers. In particular, useful X-ray diffraction[3,4] and electron spin resonance[5] measurements have been made on such systems. It is of interest to know whether such studies can be extended to other lipid classes and to seek correlations between the structures formed in lipid multilayers and other three-dimensional structures. This paper presents structural information derived from multilayers formed by homologous series of glycerides, phosphatidylethanolamines and phosphatidylcholines.

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Furthermore, in view of the current interest in the structures of lipoproteins and biological membranes, it is important to have direct information on the conformations adopted by proteins at interfaces. Another aim of this work has been to investigate the structure of some polypeptide and protein multilayers produced by deposition methods.

EXPERIMENTAL

Materials

(i) *Lipids.* The synthesis of the saturated 1,2-diacyl-L-lecithins and phosphatidylethanolamines used in this study, with the exception of 1,2-dipalmitoyl phosphatidylethanolamine, have all been described previously[6]. The latter compound was purchased from Fluka AG Ltd (Buchs, Switzerland). The chromatographically pure N–CH_3-substituted distearoyl-L-phosphatidylethanolamines and distearoyl-L-phosphatidylserine were synthesized and generously provided by Mr J.S. Chadha of this laboratory. Synthetic distearoyl-L-phosphatidic acid was a gift from Dr A. Davies of this laboratory. The saturated 1,2- and 1,3-diglyceride homologues were synthesized in this laboratory and were chromatographically pure. The cholesterol was obtained from British Drug Houses Ltd, and recrystallized until it gave a single spot on thin-layer chromatographic analysis.

(ii) *Polypeptides.* Poly-L-alanine and poly-L-valine, both of high but unspecified molecular weights, were purchased from Sigma London Chemical Co. (London, S.W. 6, Great Britain). Poly-β-benzyl-L-aspartate (M_r 130000) was obtained from the same supplier. Poly-γ-benzyl-glutamate (M_r 300000) was purchased from Koch-Light Laboratories (Colnbrook, Bucks, Great Britain). A further high molecular weight preparation of the same material was generously provided by Mr W. E. Hanby of Courtaulds Ltd, (Coventry, Great Britain).

(iii) *Proteins.* Horse heart cytochrome *c* (Type VI) and lysozyme (Grade 1, 3× crystallized) were purchased from Sigma Chemical Co. β-Casein and *n*-decanoyl-β-casein have been characterized previously[7] and were generously supplied by Dr L. Irons.

Multilayer formation

The essential details of the formation of Langmuir–Blodgett multilayers have been reviewed by Gaines[2]. The methods used to form insoluble lipid monolayers at the air–water (pH 5.5) interface have been described before[6]. The water was contained in a perspex trough, the edges of which had been coated with paraffin wax, in order to maintain a suitable meniscus. The water surface was cleaned by sweeping with a teflon barrier and impurities removed by suction. The water surface was divided by a cotton thread which was anchored to the edges of the trough. The thread had been made hydrophobic by coating it with a purified paraffin grease. The trough was enclosed with an opaque cover to keep out dust and to prevent darkening of the AgCl plates which were used when infrared spectra were to be obtained. The whole apparatus was maintained at 22 ± 2 °C.

The lipid, polypeptide or protein was spread from a suitable spreading solvent on one side of the cotton thread at the air–water interface. After all the organic solvent had evaporated, the monolayer was compressed by adding a piston oil[8] to the other

side of the thread. In this way, constant surface pressures of about 10 dynes/cm (tritolyl phosphate from Hopkins and Williams Ltd, Chadwell Heath, Essex, Great Britain), 15 dynes/cm (pure triolein) and 30 dynes/cm (pure oleic acid) were achieved and maintained. The hydrophobic cotton thread prevented the piston oil from contaminating the monolayer under investigation.

Small glass or teflon plates (2 cm × 1 cm) which had been cleaned in chromic acid and thoroughly rinsed with triple-distilled water were clamped to the teflon arm of a mechanical dipper which overhung the insoluble monolayer. The arm of the dipper was raised by a cam action so that a 2-cm plate was completely withdrawn through the monolayer-covered interface in about 10 min. The precise withdrawal time was varied according to the film that was being picked up. The arm dropped under its own weight so that the plate moved down through the monolayer in about 1 s. The cam was driven by an electric motor so that the dipper could be left running continuously until the multilayer forming on the glass or teflon support reached the requisite thickness. For the hydration studies, the lipid multilayers were swollen by exposing them to an atmosphere of very high relative humidity.

Oriented multilayers were formed more quickly by simply placing a solution of the material under investigation on the support and allowing the solvent to evaporate[4]. This method of formation was used in selected cases.

Measurements of multilayers

(i) *X-ray diffraction.* Multilayers at least 100 monolayers thick were formed on either glass or teflon supports and examined using a Philips X-ray powder diffractometer. Diffraction was recorded in the range $d = 1.5$–60 Å. CuK_α radiation was obtained by the use of a Niβ-filter and a pulse height analyzer. In order to obtain X-ray diffraction measurements over a temperature range (20–150 °C), certain phospholipid multilayers were placed in an electrical heating block mounted in the sample chamber of the diffractometer. Several films of poly-γ-benzylglutamate were removed from the solid support, rolled into small spheres and their diffraction patterns recorded photographically using a 6-cm Unicam S25 powder camera.

(ii) *Infrared spectroscopy.* Multilayers consisting of 400 monolayers were formed on both sides of AgCl plates and placed in the light path of a Grubb Parsons (GS4) double-beam grating spectrophotometer and their adsorption spectra obtained over the wavelength range 2–15 μm.

RESULTS

(i) *Lipid multilayers*

The monolayer characteristics, at the air–water interface, of the saturated phosphatidylcholines and phosphatidylethanolamines used in this study have been described previously[6]. Diglycerides can form films in all the usual monolayer states[9] although 1,2-dipalmitin monolayers are fully condensed at room temperature (Phillips, M.C., unpublished). The force–area curves of 1,3-dipalmitin at various temperatures have been described[10]. 1,3-Distearin forms fully condensed monolayers at room temperature whereas 1,3-dimyristin monolayers at room temperature are only fully condensed at high film pressures. In general, these lipids only deposited readily on the solid support when the surface pressure was high enough to ensure that the lipid mono-

layer at the air–water interface was fully condensed. One of the three piston oils was selected to optimise the deposition and still give stable lipid monolayers at the air–water interface.

With the deposition method used in this work, the lipid monolayer transferred from the air–water interface to the solid support only as the plate descended through the water interface. The multilayers were therefore "X-type" using the Blodgett–Langmuir nomenclature[1]. The multilayers were usually built up until they contained 100–200 monolayers. Several orders of the principal X-ray spacing corresponding to the bimolecular lamellar repeat unit were observed with such films. Since the number of diffraction lines obtained under equivalent conditions from different multilayer films of equivalent thickness varied considerably, it appears that the degree of molecular orientation within the films depends upon both the lipid class and, within a given class, upon the hydrocarbon chain length. The phosphatidylethanolamines deposited to give extremely regular multilayers and up to fifteen orders of the lamellar repeat unit were recorded (see Fig. 1). The spacings between 3–6 Å, corresponding to the diffraction from lattice planes normal to the planes of the lamellae, which give information on the mode of hydrocarbon chain packing, were not observed due to restrictions imposed by the diffraction geometry.

The lamellar long spacings for the homologous series of 1,3-diglycerides and 1,2-phosphatidylcholines and phosphatidylethanolamines in the anhydrous state are shown in Fig. 2. The long spacings exhibited by multilayers of 1,3-dipalmitin, 45 Å, and 1,2-dipalmitin, 46 Å, are similar to values given by Stenhagen[11]. The long spacings for a series of glyceride molecules containing two stearic acid chains and different polar groups are listed in Table I. No changes in the diffraction spacings were observed when glyceride multilayers were exposed to an atmosphere of high relative humidity.

For the two classes of phospholipids studied the lamellar diffraction spacings are clearly chain length dependent. When the X-ray diffraction pattern was recorded as a function of temperature in the range 20–150 °C a reduction in the long spacing was observed at temperatures corresponding to the hydrocarbon chain melting transition[12,13]. Levine *et al.*[3] have described the formation of multilayers of dipalmitoyl lecithin and have shown that the lamellar spacing of 58 Å in the dry state increases on increasing hydration. Our results confirm this behavior but of the lipids studied only the lecithin multilayers showed swelling in an environment of high relative humidity.

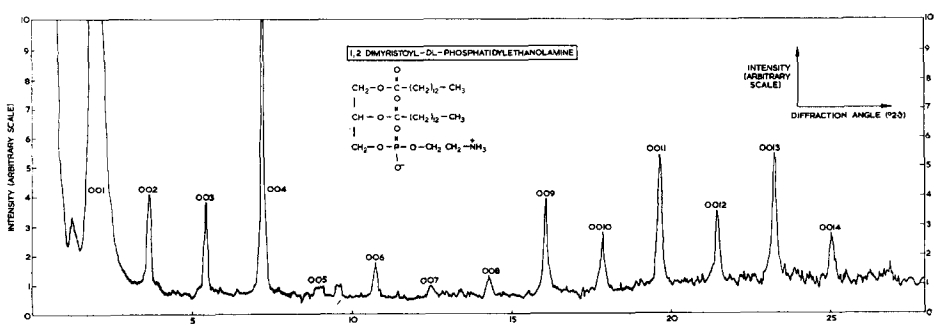

Fig. 1. X-ray diffraction pattern of multilayer of 1,2-dimyristoyl-DL-phosphatidylethanolamine.

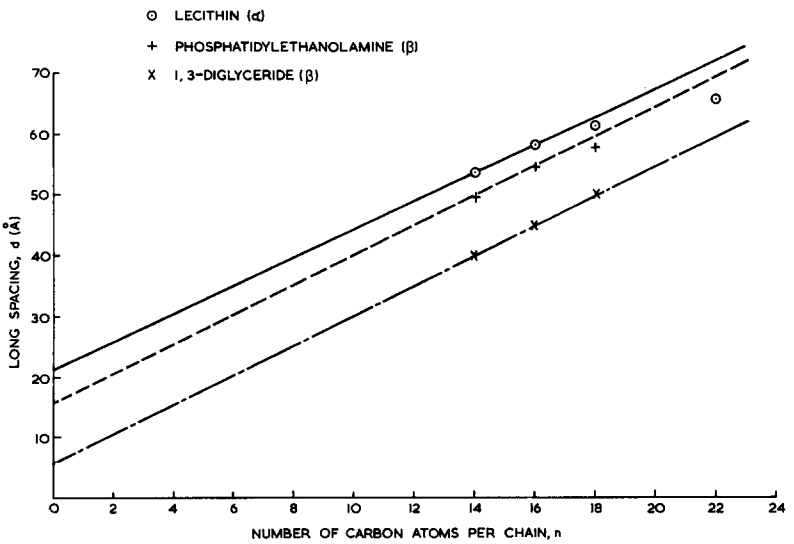

Fig. 2. Plot of long spacing *d versus* hydrocarbon chain length *n*. (A) phosphatidylcholines (α): ———, powder diffraction data; ⊙, multilayers. (B) phosphatidylethanolamines (β): - - - - - - - powder diffraction data; +, multilayers. (C) 1,3-diglycerides (β): –·—·–, powder diffraction data; ×, multilayers.

The L-stereoisomer and the DL-mixture of dimyristoyl phosphatidylethanolamine gave multilayers with identical long spacings.

Cholesterol monolayers can be deposited to give multilayers with a long spacing of 33.8 Å. This is excellent agreement with an earlier estimate[14] of 17.4±0.4 Å for the thickness of a cholesterol monolayer and a long spacing of 33.75 Å measured from a low-angle X-ray powder diffraction photograph. Cholesterol forms mixed monolayers with phospholipids[15] and multilayers containing the two compounds can be produced[4]. Multilayers of cholesterol, dimyristoyl phosphatidylethanolamine and their mixture gave infrared spectra when the films were 200 bilayers thick. The spectrum of the dimyristoyl phosphatidylethanolamine multilayer showed no OH absorption at 2.8–3.0 μm indicating that it was anhydrous and that water is not retained during the

TABLE I

d_{100} FOR A SERIES OF DIACYL LIPIDS CONTAINING STEARIC ACID CHAINS

Lipid	*Multilayer* d_{100} (Å)	*Crystal* d_{100} (Å)	*Crystal form*
1,3-Distearin	50	50	β
1,2-Distearoyl phosphatidic acid	58	58	α
1,2-Distearoyl phosphatidylethanolamine	58	60	β
1,2-Distearoyl-*N*-methyl phosphatidylethanolamine	60	—	—
1,2-Distearoyl-*N*-dimethyl phosphatidylethanolamine	62	—	—
1,2-Distearoylphosphatidylserine	60	62	α
1,2-Distearoyllecithin	62	63	α-monohydrate

film formation. The OH group in the cholesterol multilayer appears to be hydrogen bonded because the absorption was at 2.96 μm rather than the 2.8 μm characteristic of free OH. This absorption is unchanged in the mixed dimyristoyl phosphatidylethanolamine/cholesterol multilayers so we were not in a position to make comparisons with earlier infrared studies[16,17] of hydrogen bonding between cholesterol and egg lecithin.

The nature of the substrate does not seem to play an important role in determining the structure of the multilayer. Presumably, the orientation of the first monolayers on glass and teflon are reversed with the polar groups oriented towards the hydrophilic glass and the terminal methyl groups of the lipid chains orientated towards the hydrophobic teflon. However, the long spacings derived from lipid films on both solids are identical. In the studies described here, glass was preferred as the solid support since teflon gives an intense diffraction line which sometimes overlapped with diffraction lines from the lipid. The method of formation does not affect the molecular structure of the multilayers since the long spacings were identical for films formed by the Blodgett–Langmuir method and by evaporation from an organic solvent onto the solid support. However, for the evaporation method the observed X-ray diffraction was more diffuse presumably indicating some decrease in the long-range order.

(ii) *Polypeptide and protein multilayers*

Poly-γ-methyl-L-glutamate monolayers at the air–water interface have been well characterized and force–area curves are available in the literature[18–20]. When the spreading solvent contains an excess of chloroform, the polypeptide adopts an α-helical conformation at the interface[18] whereas the β-conformation occurs when pyridine-rich solutions are used[20]. Helices are present in monolayers of poly-β-benzyl-L-aspartate[21,22] and poly-γ-benzyl-L-glutamate[18,23]. Poly-L-alanine adopts a predominantly α-helical conformation in monolayers whereas poly-L-valine forms an extended chain structure[24].

X-type multilayers of these polypeptides were formed on glass by the method described above for the lipids. Monolayers of some of the polypeptides could not be transferred from the air–water interface by the dipping technique and in these cases the method involving evaporation from an organic solvent was utilized. These multilayers were more disordered than those formed by the Blodgett–Langmuir technique and occasionally no sharp diffraction lines were observed.

Poly-γ-benzylglutamate formed the most ordered multilayers and three diffraction lines at 12.3 ± 0.2 Å, 7.1 ± 0.1 Å and 6.1 ± 0.05 Å were observed (see Fig. 3A). The ratio of these spacings is $1:1/\sqrt{3}:1/\sqrt{4}$ indicating the presence of hexagonal symmetry within the structure. There is, of course, an insufficient number of reflections to allow a detailed structural investigation. However, the d_{100} spacing is similar to that observed by Malcolm[18] with collapsed monolayers of the same polymer. The observed spacings correspond to an hexagonally packed array of rod-like molecules with an axis to axis distance of 14.2 Å and if the polypeptide is in the α-helical conformation this distance corresponds to the helix axis–axis separation. In confirmation of this, higher orders of this hexagonal diffraction pattern were observed photographically from a rolled up fragment of the multilayer. More than 15 diffraction lines were observed and these were similar to those obtained by Bamford *et al.*[25] for fibres of α-helical poly-γ-benzylglutamate. Since it is known that treatment of polypeptide fibres with formic acid gives the β-conformation[26], poly-γ-benzylglutamate multilayers

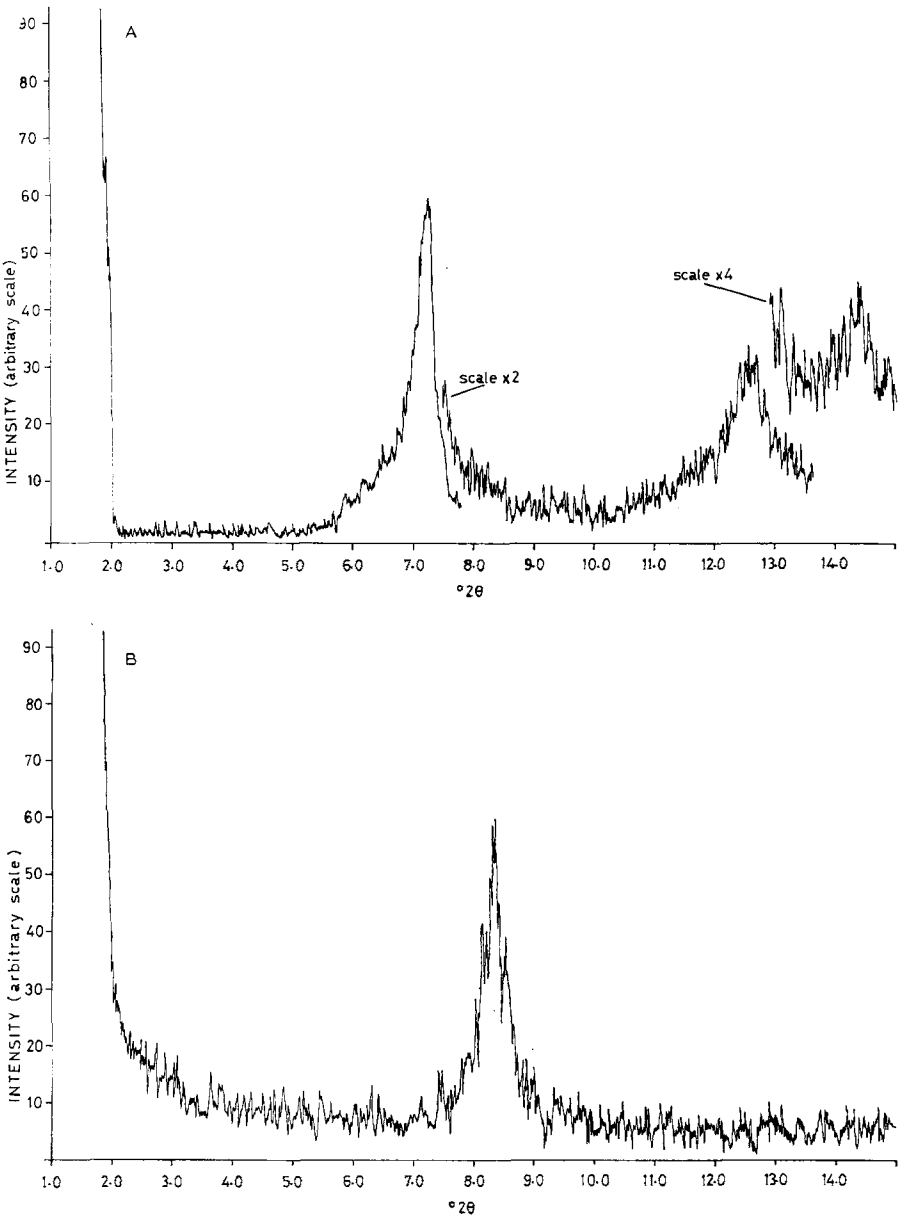

Fig. 3. X-ray diffraction pattern of polypeptide multilayers (A) poly-γ-benzyl-L-glutamate, (B) poly-γ-methyl-L-glutamate.

were soaked in 98% formic acid in attempt to promote the α-helix to β-sheet structural transition. However, the observed effect on the X-ray diffraction pattern was minimal, the diffraction lines becoming weaker and were diffuse. No diffraction lines consistent with the presence of a β-sheet structure were observed.

Only one diffraction line of low intensity was usually observed with poly-γ-

methyl-L-glutamate multilayers (see Fig. 3B), irrespective of whether they were formed by the Blodgett–Langmuir method or by solvent evaporation. From chloroform, formic acid and pyridine solutions, this polypeptide gave a diffraction spacing of 10.5 ± 0.2 Å. Since only one diffraction line was observed little structural information can be derived except to suggest that it arises from the molecular stacking normal to the surface of the support. In two cases, further orders were observed and these indicated that this polymer was also adopting an hexagonal packing of α-helices. Attempts to change the secondary structure to β-sheet by spreading from a different solvent[20] or by using low molecular weight material (M_μ 5400) were unsuccessful as judged by the invariance of the diffraction spacing. Exposing the multilayers to atmospheres of high relative humidity did not give rise to significant changes in the observed lattice dimensions.

One diffraction line at 13 Å was observed for multilayers of poly-γ-benzyl-L-aspartate which could only be formed by evaporation. Poly-L-valine multilayers gave a spacing of 9.4 Å but we were unable to observe any diffraction from poly-L-alanine films. In all cases, the deposited films are presumably insufficiently ordered to give higher order diffraction lines or more informative diffraction patterns and thus further structural characterization of these multilayers was not possible.

The monolayer characteristics of proteins at the air–water interface have been reviewed by James and Augenstein[27]. Lysozyme[28] and β-casein[29] have been particularly thoroughly investigated. Despite the fact that the surface properties of these two proteins are very different the lattice spacing of their multilayers is the same at 9.3 Å. The same spacing is obtained from cytochrome *c* and *n*-decanoyl-β-casein multilayers. This spacing is close to an earlier value of 9.5 Å for the thickness of protein monolayers in ovalbumin multilayers[30,31].

DISCUSSION

Lipid multilayers

In Fig. 2 the principal long spacings obtained from randomly oriented polycrystalline (powder) samples of the various lipids are plotted as a function of chain length. The long spacings from the multilayer preparations are shown as the individual points and clearly the correspondence for most of the lipids indicates that the anhydrous multilayers can be regarded as oriented "crystals". The various polymorphs of these lipids give rise to different long spacings[12,32,33,35]. The short spacings which characterize different polymorphic forms of crystals of lipids were not observed because of the particular diffraction geometry used and thus we cannot assign crystal types on this basis. However, the correlations of long spacings in Fig. 2 indicate that the 1,3-diglycerides and 1,2-phosphatidylethanolamines assume the β-type packing of their hydrocarbon chains in the stacked multilayer system (for a discussion of the nomenclature of lipid chain packing, see ref. 33). On the other hand, the hydrocarbon chains of the short-chain phosphatidylcholine homologues seem to be packed in an hexagonal lattice (α-form) as the monohydrates. The decreased lattice dimension observed with the longer chain length dibehenoyl phosphatidylcholine multilayer occurs because the enhanced chain interaction causes the molecules to pack as the anhydrous β-crystal. The crystal form adopted by the lipid molecules defined in Fig. 2 and Table I depends upon the net magnitudes of the apolar chain interaction energy

and polar headgroup interaction energy; the β-form has the lower potential energy. The resultant magnitude is affected by the length of the hydrocarbon chains and the size of the polar groups. The β-form is favored by a high chain–chain interaction energy and occurs when the cross-sectional area of the molecule is determined by the chains and van der Waals interactions are maximized. Thus the lipids with smaller headgroups can readily crystallize into the β-form whereas as the headgroup size is increased (for a given chain length, see Table I) there is an increasing propensity for the α-form to be adopted. Any increase in the attractive interaction energy in the polar group lattice will also favor the β-form. For the phospholipids which are zwitterionic, obviously the conformation of the headgroup can greatly influence this energy.

Both the multilayer preparations and the polycrystalline powder samples show good agreement in terms of their lamellar repeat distance *d*, the relative intensities of the low angle reflections and their melting behavior, particularly for the C_{14}, C_{16} and C_{18} homologues. From this we would predict that the molecular packing of the hydrocarbon chains and the polar head groups, and their conformation, would be identical in the two cases. However, without complementary and confirmatory diffraction data at wide angles a more extensive treatment of the multilayer data is not justified. Thus although it seems probable that the head group conformations predicted from model building studies based on the space available to the polar head group[34], will be applicable to the molecules in the multilayer system, more detailed electron density profiles of phospholipids in both multilayers[4] and other systems are necessary to confirm this.

Polypeptide and protein multilayers

The conformations adopted by proteins at interfaces are not known and cannot be studied directly at the air–water interface at present because of the lack of suitable techniques which have sufficient sensitivity. Formation of stacked arrays of protein monolayers offers an opportunity to overcome the latter problem. Our aim has been to investigate the roles of secondary structure by using homopolypeptides which adopt well-defined structures and of tertiary structure by comparing the behavior of globular and disordered proteins.

Most of the hydrophobic homopolypeptides utilized here have a marked preference for the α-helical conformation when spread at the air–water interface[18]. When multilayers are formed from such monolayers, the monolayers deposit as sheets containing regions of aligned, intra-molecularly hydrogen-bonded, α-helical molecules. Formation of a stacked film of such sheets leads to the rod-like molecules packing with hexagonal symmetry in the three-dimensional array. The monolayer repeat spacing is dependent upon the dimensions of the helix and the length of the side chains. When multilayers are formed from monolayers containing inter-molecularly hydrogen-bonded molecules in a β-sheet conformation, the inter-sheet repeat distance will be determined by the side-chain spacing. The spacings given above for the polypeptide multilayers tend to reflect the size of the side chains; as the chain becomes longer the repeat distance increases. For example, the inter-sheet spacing of poly-L-lysine hydrochloride in the β-pleated sheet structure is 15 Å (ref. 36).

Although the tertiary structure of proteins plays a dominant role in determining the surface activity of proteins, the diffraction spacings of multilayers of lysozyme (globular and rigid) and β-casein (random and flexible) are the same. Also, the helix contents of these two proteins are quite different; lysozyme contains about 40% helix

whereas β-casein contains no helix. The invariant monolayer repeat distance for the various protein multilayers is consistent with there being a common structure in every case. It is known that virtually all tertiary structure can be destroyed by the unfolding and reorientation which occurs during the establishment of equilibrium at the air–water interface[28]. Although the above data for multilayers of the hydrophobic homopolypeptides indicate that long helical regions are stable at the air–water interface and in the multilayer, short regions of helix adjacent to random coil segments in a protein molecule may unfold more easily. In situations where this occurs, the structure of the monolayer at the air–water interface at high areas per protein molecule will be a two-dimensional array of amino acid residues. If the proteins we have studied have flattened into the interface to give this structure, then the 9.3-Å repeat distance in protein multilayers is presumably a reflection of the mean side-chain length of the amino acid residues which make up the macromolecule. The substitution of *n*-decanoic acid residues onto the lysine side chains of β-casein did not result in a detectable change of the spacing. However, this modification will only alter the length of about 15 of the 209 residues (*i.e.* 7%) in the β-casein molecule. The mean size of the side chains in β-keratin leads to a 9.8-Å separation of the hydrogen-bonded sheets[37].

In summary, the X-ray diffraction data from highly ordered multilayers of different lipid molecular species and a study of their chain length dependence has enabled us to demonstrate the complex polymorphism of lipids characteristic of other three-dimensional arrays of lipids and to suggest structural features, particularly the hydrocarbon chain packing, of the individual lipid classes. It would appear that the multilayer preparations of polypeptides and proteins are less ordered, with the packing of α-helical rods of the homopolypeptides being the most stable structures. The diffraction data from multilayers of proteins are consistent with an unfolding of globular proteins when absorbed at the air–water interface. In view of the importance of lipid–protein interactions in fundamental biological structures such as membranes and serum lipoproteins, an obvious continuation of this multilayer approach will be a study of the molecular arrangement of lipoproteins formed at monolayer surfaces.

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