BBA 75368

THE INTERACTION BETWEEN CYTOCHROME *c* AND PURIFIED PHOSPHOLIPIDS

C. J. HART, R. B. LESLIE, M. A. F. DAVIS AND MISS G. A. LAWRENCE
Unilever Research Laboratory, Colworth/Welwyn, The Frythe, Welwyn, Herts (Great Britain)
(Received August 4th, 1969)

SUMMARY

Isooctane-soluble complexes formed between cytochrome *c* and various mixtures of phosphatidylserine and phosphatidylcholine have been studied, with the following results.

1. For a given phospholipid mixture there is a unique total lipid to protein ratio for maximum complex extraction.

2. At maximum extraction, each cytochrome *c* molecule binds 9 molecules of phosphatidylserine and 5–60 molecules of phosphatidylcholine according to the composition of the original phospholipid mixture. When the phosphatidylserine to cytochrome *c* ratio exceeds 9:1, decreased extraction of both lipid and protein is observed.

3. Other saturated paraffins exhibit similar behaviour to isooctane, but unsaturated, or halogenated hydrocarbons, do not extract complexes under similar conditions.

4. In 100 mM NaCl, each cytochrome *c* molecule is associated with 70–100 phosphatidylserine molecules, and a maximum of 400–500 phosphatidylcholine molecules.

The results suggest an electrostatic interaction between the phosphatidylserine and protein. The mode of binding of the phosphatidylcholine is not clear.

INTRODUCTION

The application of physical techniques to study the interaction between lipids, particularly phospholipids, and proteins in membranes and serum lipoproteins has increased significantly in recent years[1–8]. Similar studies with well-characterised model complexes between phospholipids and various proteins should provide information relevant to the interaction involved in natural lipoprotein systems. Several groups of workers have studied the formation of such complexes between well-defined proteins and phospholipids[9–12]. In these studies the composition of the complexes and the various possible binding forces have been found to be dependent upon the type of protein or phospholipid involved and the conditions of formation. We have studied the interaction between cytochrome *c* and phospholipids in considerable detail, and recently presented the results of low-angle X-ray scattering studies of complexes in isooctane[13], and X-ray diffraction studies of the complexes formed in

water[14]. In this paper we present the analytical data, relevant to a complete characterization of the complexes.

MATERIALS AND METHODS

Materials

A.R. grade reagents were used without further purification. 2,2,4-Trimethylpentane (isooctane) (Hopkins and Williams) was spectroscopic grade. Paraffinic solvents were obtained from Ralph N. Emmanuel Ltd., Fluka or Kodak. Water was glass-distilled. Aluminium oxide was Woelm neutral, activity grade 1. Silicic acid was obtained from Malinkcrodt (100 mesh); silica gel G from E. Merck A.G.; cytochrome *c* (horse-heart type VI) from Sigma Chemical Co.; lysozyme from Armour Pharmaceutical Co.

Phosphatidylcholine

Yolks from one dozen fresh eggs were extracted 3 times with 500 ml acetone. The acetone-insoluble residue was extracted 3 times with 150 ml of 95 % alcohol and the combined filtrates were concentrated under vacuum to 150 ml. The phospholipids were precipitated by 300 ml of acetone–water (1:1, v/v), collected and dissolved in 50 ml ether. The phospholipids were precipitated twice more by 125 ml acetone and the final precipitate taken up in chloroform–methanol (3:2, v/v) and mixed with 15 g silicic acid. After filtration, the soluble extract was dried under vacuum and purified by alumina chromatography. The lipid was dissolved in benzene and eluted from an alumina column (80 g aluminium oxide, dried at 110° for 48 h, packed in a 2.5-cm-diameter column to give a bed height of 13–15 cm) with benzene–methanol (95:5, v/v). The first 300 ml eluant contained cholesterol, and were discarded. The subsequent eluant was collected until contamination with lysophosphatidylcholine was observed. The yield of chromatographically homogeneous phosphatidylcholine was approx. 3 g.

Phosphatidylserine

Brains from freshly killed oxen were stored in ice until extraction, according to the method of FOLCH[15], could be started (usually 1 h after collection). 2–3 brains (800–1000 g) were normally processed together. The final "cephalin" powder (12 g) was fractionated to give Folch Fractions 1, 3 and 5. Fraction 1 was usually treated a second time to yield an additional Fraction 3. Fraction 3 was 90–95 % phosphatidylserine and was further purified by DEAE-cellulose (Whatman D.E. 32) chromatography by the method of ROUSER AND FLEISCHER[16]. The yield was 1–2 g after conversion to the monosodium salt by the method of PAPAHADJOPOULOS AND MILLER[17].

Characterization

The phospholipids were identified by thin-layer chromatography using silica gel G as the support phase. The chromatograms were developed with either chloroform–methanol–7 M aq. ammonia (230:90:15, by vol.) or chloroform–methanol–water (65:25:4, by vol.). Free amino groups were detected by spraying with ninhydrin and heating at 100° for 5 min. Phosphate groups were detected by spraying with molybdenum blue reagent. The presence of other material was detected by charring the plate at 120° after spraying with molybdenum blue reagent. A sample of the

phosphatidylserine was examined by gas–liquid chromatography. The fatty acid distribution was similar to that reported by PAPAHADJOPOULOS AND MILLER[17].

Preparation of isooctane-soluble complexes

The procedure was essentially that of DAS *et al.*[10,11]. A chloroform solution of the phospholipids was evaporated to dryness and then shaken for 10 min on a wrist action mechanical shaker with water to give a 0.1 % dispersion. Required amounts of this dispersion were then mixed in a 50-ml volumetric flask with 0.5 mg of cytochrome *c*, or lysozyme, in aqueous solution, to a total aqueous volume of 4 ml. Ethanol (2 ml) and isooctane (4 ml) were added, and the flask shaken in a reciprocal shaker at 250 strokes per min with an amplitude of 3 cm, for 30 min. The isooctane layer was separated by centrifugation at 3000 × *g* in a bench centrifuge for 5 min and removed for analysis.

Analysis of isooctane-soluble complexes

Cytochrome *c* was determined by the absorbancy of the complex in isooctane at the isosbestic point at 410 mμ; the molar extinction was taken to be $10.61 \cdot 10^4$ (ref. 18). Lysozyme was determined by the method of LOWRY *et al.*[19] after evaporation of the isooctane and re-solubilisation in 2 % Na_2CO_3 in 0.1 M NaOH. Total lipid phosphorus was determined by the method of CHEN *et al.*[20]. Phosphatidylcholine to phosphatidylserine ratios in the complex were determined by quantitative thin-layer chromatography.

The chromatograms were developed with chloroform–methanol–7 M aq. ammonia solvent, sprayed with Rhodamine 6 G, viewed under ultraviolet light, and the position of each fluorescent spot marked. The silica gel G containing each spot was then transferred into a tube and extracted twice with 2 ml of 1 M methanolic HCl at 60° for 15 min. The extracts, combined after centrifugation, were evaporated to dryness for phosphorus determination.

Interactions in aqueous solution

The turbidity of an aqueous suspension of phospholipids and cytochrome *c* was measured as a function of pH using an E.E.L. nephelometer. The binding capacity of cytochrome *c* for the phospholipids was determined by titrating a solution of cytochrome *c* with a concentrated phospholipid dispersion. After each addition of phospholipid, the precipitated complex was centrifuged at 5000 × *g*, and the amount of cytochrome *c* reacting determined by the change in absorbance (measured in an E.E.L. colorimeter with an Ilford 621 filter) of the clear supernatant. The absorbance readings were corrected for dilution.

RESULTS

The control experiments, namely the extraction of phospholipid into isooctane as a function of the phosphatidylserine content of a phosphatidylserine–phosphatidylcholine mixture showed that the presence of the acidic lipid (phosphatidylserine) at the 5 % level, led to a complete inhibition of the extraction of phospholipid (*cf.* ref. 10). Presumably in the mixed lipid dispersions, the charged groups of the phosphatidylserine are exposed to the aqueous phase, and this gives the dispersion a preferred water solubility.

When co-dispersions of phosphatidylserine and phosphatidylcholine were shaken with isooctane in the presence of various cations, some extraction into the organic phase was observed, though the experiments were poorly reproducible. A small amount of lipid extraction was observed in the presence of monovalent ions, but generally increased amounts were formed with the polyvalent ions (Ca^{2+}, Fe^{3+}, Sn^{4+}). When phosphatidylserine alone was shaken with $CaCl_2$, little or no extraction into isooctane was observed.

To avoid complications caused by the presence of polyvalent cations, the phosphatidylserine preparations were acid-washed before conversion to the monosodium form.

An example of the extraction of cytochrome *c* and phospholipid into isooctane with increasing phospholipid to cytochrome *c* ratios, at constant cytochrome *c*, is shown in Fig. 1. The corresponding variation of total lipid to protein, phosphatidylcholine to protein, phosphatidylserine to protein ratios for the extracted complex are also given. As can be seen, there is a unique total phospholipid to protein ratio in

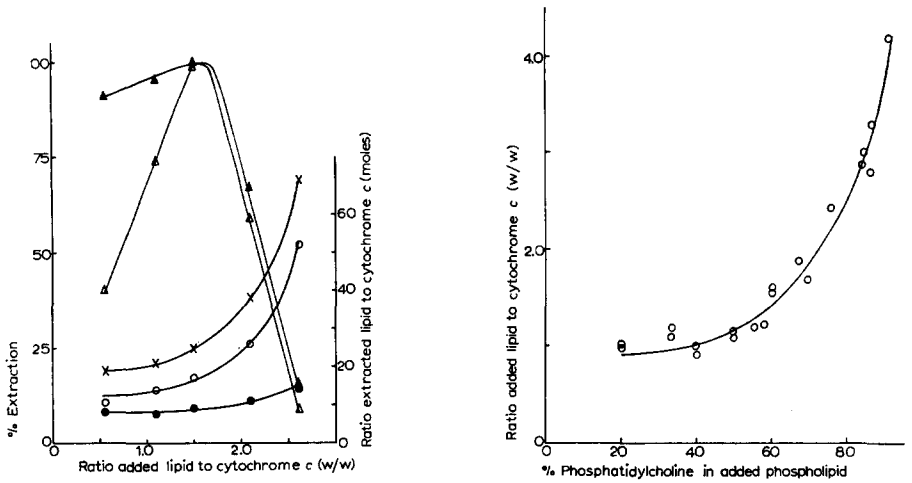

Fig. 1. A typical example of the extraction of cytochrome *c* and a phosphatidylcholine–phosphatidylserine mixture (2.3:1, w/w) into isooctane; △—△, the extraction of cytochrome *c*; ▲—▲, the extraction of phospholipid; ×—×, the total phospholipid to cytochrome *c* ratio in the complex; ○—○, the phosphatidylcholine to cytochrome *c* ratio in the complex; ●—●, the phosphatidylserine to cytochrome *c* ratio in the complex.

Fig. 2. The ratio of total phospholipid to protein in the reaction mixture required for maximum extraction, as a function of the ratio of phosphatidylcholine to phosphatidylserine.

the reaction mixture which leads to maximum extraction of the cytochrome *c* and phospholipid. This ratio in turn depends on the ratio of phosphatidylcholine to phosphatidylserine used. From a series of experiments, such as those illustrated in Fig. 1, the total phospholipid to protein ratios giving maximum extraction were determined. These ratios are plotted against the phosphatidylcholine to phosphatidylserine ratios used in the experiments (Fig. 2). With phosphatidylcholine to phosphatidylserine ratios less than 1.5:1, complete extraction of cytochrome *c* is not obtained. The stoichiometry of the complexes at maximum extraction is shown in

Fig. 3, which also shows the stoichiometry at half maximal extraction *i.e.* where the phospholipid exceeds that required for maximum extraction.

The extraction characteristics and composition of a lysozyme–phospholipid complex were studied for a single phosphatidylcholine to phosphatidylserine ratio (3:2, w/w) and are presented in Fig. 4.

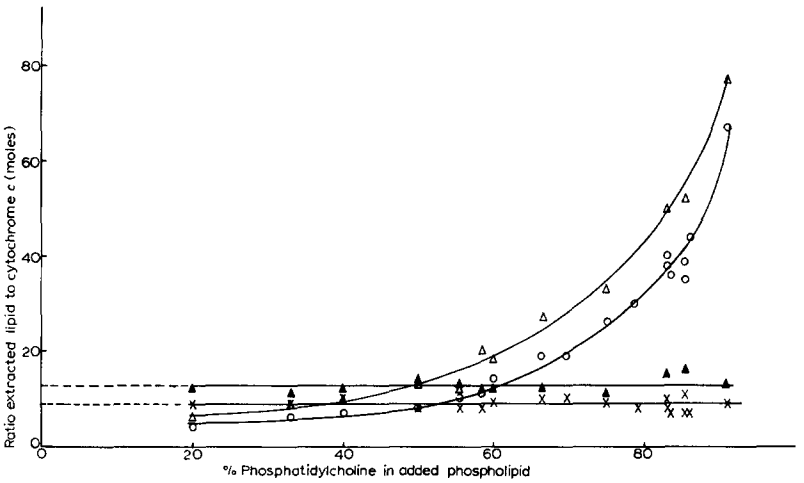

Fig. 3. The dependence of the final composition of the cytochrome *c*–phospholipid complex on the ratio of phosphatidylcholine to phosphatidylserine used. ×—×, ○—○, ratios of phosphatidylserine and phosphatidylcholine, respectively, to cytochrome *c* at maximum extraction. ▲—▲, △—△, ratios of phosphatidylserine and phosphatidylcholine, respectively, to cytochrome *c* at half maximal extraction (see text).

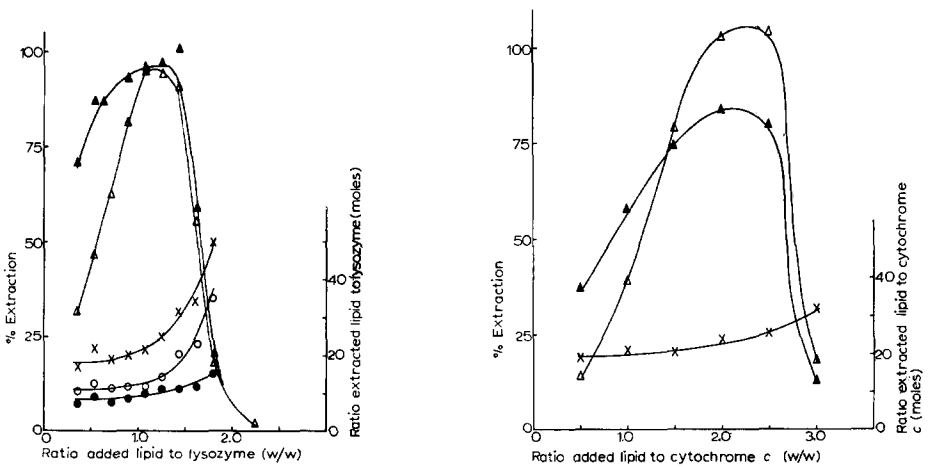

Fig. 4. The extraction of lysozyme and a phosphatidylcholine–phosphatidylserine mixture (3:2, w/w) into isooctane: △—△, the extraction of cytochrome *c*; ▲—▲, the extraction of phospholipid; ×—×, the total phospholipid to cytochrome *c* ratio in the complex; ○—○, the phosphatidylcholine to cytochrome *c* ratio in the complex; ●—●, the phosphatidylserine to cytochrome *c* ratio in the complex.

Fig. 5. The extraction of cytochrome *c* and a phosphatidylcholine–phosphatidylserine mixture (3:1, w/w) into pentane. △—△, the extraction of cytochrome *c*; ▲—▲, the extraction of phospholipid; ×—×, the total phospholipid to cytochrome *c* ratio in the complex.

Extraction and solubility of phospholipid–cytochrome c complexes in other solvents

Various saturated paraffins, other than isooctane were able to extract apparently similar complexes (see Table I, and Fig. 5), though *n*-heptane and 2,4-dimethylpentane behaved anomalously. The unsaturated and halogenated paraffins did not extract complexes. By evaporating the isooctane under a stream of N_2 until just dry, the lipid and protein could be quantitatively transferred to other solvents such as chloroform, benzene, ether and tetrachloroethylene. In the case of benzene and tetrachloroethylene, ultracentrifugation and NMR studies indicated that the complex was dissociated.

TABLE I

THE EXTRACTION OF CYTOCHROME *c* AS A PHOSPHOLIPID COMPLEX INTO VARIOUS SATURATED, UNSATURATED AND HALOGENATED PARAFFINS

Two parts of a phosphatidylcholine–phosphatidylserine mixture (3:1, w/w) was reacted with 1 part (w/w) of cytochrome *c* under standard conditions (see text). The number of experiments performed is shown in parentheses.

	% of total cytochrome c extracted	*Ratio total lipid to cytochrome c*	*Ratio phosphatidyl-serine to cytochrome c*	*Ratio phosphatidyl-choline to cytochrome c*
Straight chains, saturated				
n-Pentane	110 (6)	30	9	21
n-Hexane	78 (6)	34	12	22
n-Heptane	27 (6)	approx. 140	—	—
n-Octane	81 (6)	27	10	17
n-Decane	77 (6)	—	—	—
n-Tetradecane	72 (6)	—	—	—
Branch chains, saturated				
3-Methylpentane	91 (3)	38	—	—
2,2-Dimethylpentane	110 (3)	30	8	22
2,3-Dimethylpentane	83 (6)	32	—	—
2,4-Dimethylpentane	9 (6)	approx. 400	—	—
2,2,4-Trimethylpentane*	83 (15)	32	9	23
2,2,3-Trimethylpentane	103 (3)	30	9	21
2,2,5-Trimethylhexane	77 (3)	31	—	—
2,2,4-Trimethylhexane	59 (3)	29		
Unsaturated chains				
n-Octene	7 (3)	—	—	—
2,2,4-Trimethyl-1-pentene	8 (3)	—	—	—
Halogenated chains				
1-Bromopentane	3 (3)	—	—	—
3-Chloromethylpentane	8 (3)	—	—	—
3-Bromomethylheptane	27 (3)	—	—	—

* Isooctane.

Phospholipid–cytochrome c interaction in water

The effect of pH on the turbidity of a phosphatidylserine–cytochrome *c* mixture is shown in Fig. 6. The region of maximum turbidity at pH 3.8–4.0 was chosen for the following titrimetric experiments. A typical experiment showing the quantitative

precipitation of cytochrome *c* by a phosphatidylcholine–phosphatidylserine (2:1, w/w) mixture is shown in Fig. 7. From such titrations the phosphatidylserine to cytochrome *c* and the phosphatidylcholine to cytochrome *c* ratios may be calculated. These ratios are plotted as a function of the composition of the phosphatidylcholine–phosphatidylserine mixture in Fig. 8.

Two experiments of the type shown in Fig. 7 were performed at pH 7.0 showing good agreement with those at pH 3.8 (Fig. 8). At pH 7.0, however, it was sometimes found that the complex resisted centrifugation at low speeds (5000 rev./min) but this was overcome by addition of NaCl to give a 100-mM solution.

It was possible to extract into isooctane, a phospholipid–cytochrome *c* complex from the precipitated material. (The precipitated complex had a ratio of phospholipid

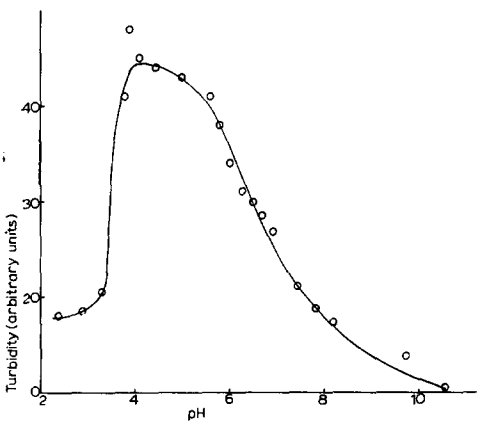

Fig. 6. The turbidity of a phosphatidylserine–cytochrome *c* mixture in water as a function of pH.

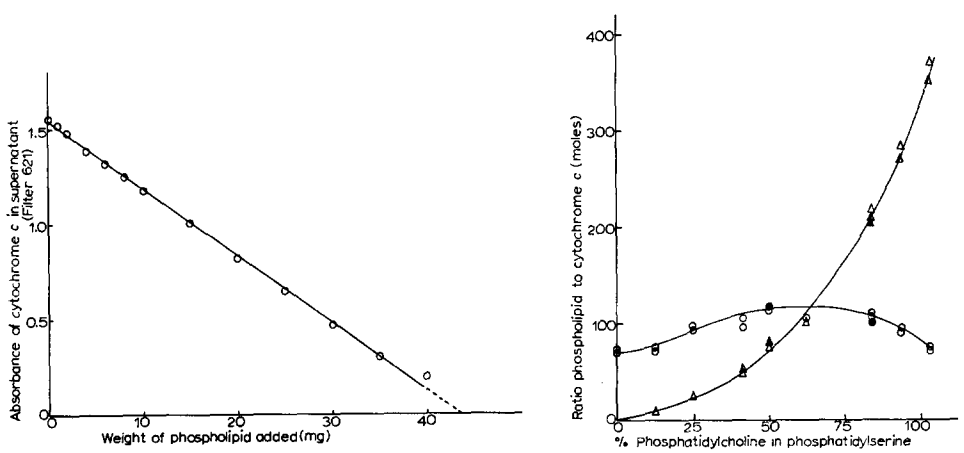

Fig. 7. A typical titration of cytochrome *c* by a phosphatidylcholine–phosphatidylserine mixture (2:1, w/w).

Fig. 8. The binding of phospholipids to cytochrome *c* in water as a function of per cent phosphatidylcholine in a phosphatidylcholine–phosphatidylserine mixture. O—O, phosphatidylserine to cytochrome *c* ratio and △—△, phosphatidylcholine to cytochrome *c* ratio, at pH 3.8. ●, phosphatidylserine to cytochrome *c* ratio and ▲, phosphatidylcholine to cytochrome *c* ratio at pH 7.0 (see text).

to protein of 200:1 or more.) However, the isooctane extract was turbid, indicating the presence of considerable excess lipid. Repeated washing with alcohol-water (1:2, v/v), removed phospholipid, clarifying the isooctane solution, and leaving a complex with a phospholipid to protein ratio in the range 20–30:1.

DISCUSSION

An interaction between cytochrome *c* and various phospholipids has been demonstrated by a number of workers[1,10,11], but the details of the interaction and the structures of the resulting complexes are not understood. Considerable conflict exists in the literature[10,21,22], concerning the ratio of molecules of phospholipid bound per cytochrome *c* molecule, reported values having varied between 8:1 and over 100:1. In earlier work phosphatidylcholine apparently formed high-ratio, isooctane-soluble complexes[22], but the later work with highly purified phosphatidylcholine, indicated that this material was in fact unable to form an isooctane-soluble complex[10].

In a series of very careful studies with highly purified phospholipids, DAS *et al.*[10] found that the complexes formed when mixed phospholipid dispersions were used, appeared to have preferred stoichiometries of 22 or 32 phospholipid molecules per protein molecule. These authors' interpretation was that the mixed phospholipids produce complexes in which the number of phospholipid molecules bound to the protein approaches the available number of charged sites on the cytochrome *c*, and that the major binding forces were therefore electrostatic in origin.

The amino acid sequence of cytochrome *c* is now known, and there are 9 polar areas containing 2 or more charged lysine residues. There are also 8 clusters of non-polar residues[18]. For steric reasons only one acidic lipid may be bound by ionic interaction to each cluster of basic residues. Thus it may not be possible for each of the 19 lysine residues to bind an acidic lipid molecule, and an alternative explanation must be sought to account for the binding of the additional phospholipid.

In conjunction with our studies of the structures of the complexes in water[14] and isooctane by physical techniques[13], we have undertaken a detailed analytical study relevant to the problems raised above.

Isooctane-soluble complexes

During these studies, it became apparent that the extraction of the complexes into isooctane, and their composition were mutually dependent upon the ratio of total lipid to protein, and on the ratio of phosphatidylcholine to phosphatidylserine. Complete extraction of the protein may be achieved in either of the following ways. If the ratio of phosphatidylcholine to phosphatidylserine is high, then complete extraction requires a relatively high total phospholipid to protein ratio. If, on the other hand, the ratio of phosphatidylcholine to phosphatidylserine is lower, then a lower total phospholipid to protein ratio will produce complete protein extraction.

A salient feature arising from this work, illustrated in Figs. 1 and 4, is that the ratio of phosphatidylserine to protein changes relatively slightly throughout the whole of the extraction curve, but that the ratio of phosphatidylcholine to protein, increases smoothly and this accounts for the overall increase of the total phospholipid to protein ratio. Thus in Fig. 1, the ratio of phosphatidylserine to cytochrome *c*, remains fairly constant at 9:1, throughout most of the extraction curve, and then increases to about

12:1 with a concomitant pronounced decrease in protein (and lipid) extraction. On the other hand, the phosphatidylcholine varies continuously between 11–50:1 giving overall stoichiometries from 20:1 to over 60:1. In other cases, although the phosphatidylcholine varied within different limits, the phosphatidylserine to protein ratio behaved similarly, remaining constant at 9:1 throughout most of the extraction curve. The binding of the 9 phosphatidylserine molecules correlates with the 9 clusters of basic residues, strongly indicating an electrostatic interaction. The presence of cations in the reaction mixture reduces complex formation, which is consistent with an electrostatic interaction (refs. 9, 11 and unpublished studies).

In the absence of phosphatidylcholine, no isooctane-soluble complexes can be formed. Thus the phosphatidylcholine is essential for extraction, but the amount required can vary between wide limits. As indicated in Fig. 3, the phosphatidylcholine to protein ratio at maximum extraction varies between 5 and 70. The higher ratios observed preclude electrostatic interactions involving the charged protein residues as being solely responsible for the binding of the additional phospholipid. The results indicate that neutralization of the protein charges is a first stage in the formation of isooctane-soluble complexes, but that additional requirements which may be satisfied by the binding of phosphatidylcholine are also important. The mode of binding of the additional phosphatidylcholine in isooctane is not clear. Interaction between the zwitterionic head group of the phosphatidylcholine molecule with charged carboxyl and lysine groups is possible, and dipole–dipole, dipole–induced dipole interactions are also possible. These interactions will be enhanced in the non-polar isooctane.

Van der Waals interaction involving the phosphatidylcholine, phosphatidylserine chains and non-polar amino acid residues are possible, but the influence of the hydrocarbon solvent may reduce the importance of such interactions.

Our recent low-angle X-ray scattering and ultracentrifuge work[13] has indicated that the complexes exist in isooctane as very large globular aggregates, and some form of protein–protein interaction is also likely to be involved. As shown in Fig. 1, addition of more phospholipid above that required for maximum extraction results in more phosphatidylserine being bound. A ratio of phosphatidylserine to protein of 13:1, invariably leads to a protein extraction only 50 % of that possible under optimal conditions as shown in Fig. 3. The additional phosphatidylserine may be bound by non-polar forces, with the result that the overall structure is modified and becomes negatively charged, and hence less isooctane-soluble.

It is of interest to compare the present results obtained with phosphatidylserine, with those of DAS *et al.*[10] using diphosphatidylglycerol (cardiolipin) and phosphatidylinositol as the acidic lipid. In their experiments the fact that both the total lipid to protein, and phosphatidylcholine to acidic lipid ratio were simultaneously varying, makes a direct comparison difficult. However, we have taken their results (Table IV of ref. 10) and expressed them in our terms, and in Table II we compare them with our results obtained under comparable experimental conditions. As may be seen the same general behaviour is exhibited in both cases. Thus there is a general increase in extraction, going through a maximum in both sets of results. Also the ratio of acidic lipid to protein is within experimental error, identical. This agreement is consistent with the interpretation that the interaction is electrostatic. The phosphatidylcholine to protein ratios show the same general trend, but differ quantitative-

TABLE II

COMPARISON OF THE EXTRACTION INTO ISOOCTANE AND LIPID TO PROTEIN RATIOS OF CYTOCHROME *c*–PHOSPHOLIPID COMPLEXES

In a, the acidic lipid was phosphatidylserine and in b phosphatidylinositol (results of DAS *et al.*[10]). The neutral lipid was phosphatidylcholine in both cases. See text for further details.

Ratio phosphatidyl-choline to acidic lipid	*Ratio added lipid to protein (w/w)*	*% of total cytochrome c extracted*		*Ratio total phospholipid to protein in extracted complex (moles)*		*Ratio acidic lipid to protein extracted*	
		a	*b*	*a*	*b*	*a*	*b*
10	0.61	20	11.4	63	86	6	8
5	0.07	30	23	30	44	8	8
2	0.83	55	29	21	30	8	10
1.67	0.88	76	28	15	25	8	10
1.43	0.95	79	47	16	23	7	10
1.25	1.00	84	33	15	23	7	10
1.00	1.10	87	8.6	15	31	8	10
0.67	1.40	40	—	20	—	13	—

ly. Further, with phosphatidylserine, a generally higher level of protein extraction was observed. These points of similarity and difference may reflect the critical dependence of the extraction and composition of the complex on the total lipid to protein ratio (Fig. 1). The differing nature of the polar group of the acidic phospholipid may also be a contributory factor.

The extraction of a lysozyme–phospholipid complex (Fig. 4) and the previously reported[10] cytochrome *c*–phosphatidylcholine–phosphatidylinositol complex suggest that similar behaviour underlies the interaction between other basic proteins and phospholipids. The attempts to extract a cytochrome *c*–phospholipid complex into other organic solvents suggest that solvent polarity may be important. Thus the non-polar saturated paraffins were efficient extracting solvents whereas the more polar unsaturated and halogenated paraffins were ineffective.

Aqueous interactions

The interaction between cytochrome *c* and phosphatidylserine in 100 mM NaCl is primarily electrostatic. Phosphatidylserine at pH 7 has a net negative charge of one and presumably binds to a region of the cytochrome *c* surface where there is a net positive charge. (Cytochrome *c* at pH 7 has 21 positively charged and 13 negatively charged amino acid residues, giving a net positive charge of 8 per molecule[18].)

Phosphatidylcholine alone causes no precipitation of cytochrome *c*. However, when mixed with phosphatidylserine, precipitation of cytochrome *c* and both lipids is observed. The salt requirement for precipitation is not absolute and probably arises from the effect of salt on lipid–water systems. Salt decreases the separation between the phospholipid bilayers (which is large in the presence of phosphatidylserine) facilitating the interaction between cytochrome *c* which has penetrated the bilayer and the phosphatidylserine.

The reduction in turbidity at high pH (Fig. 5) of a phospholipid–cytochrome *c* mixture could reflect either solubilisation or dissociation of the complex. Dissociation

at high pH might be anticipated, as the net positive charge of the cytochrome *c* is reduced. We have not yet been able to distinguish between the two possibilities.

The aqueous interaction in 100 mM NaCl is interesting as each cytochrome *c* molecule, having a net positive charge of 8 is found in association with 70–100 negatively charged posphatidylserine molecules (Fig. 7). The phosphatidylcholine is bound simply in proportion to the amount present. X-ray diffraction studies[14] have shown that the precipitated complexes possess a lamellar structure, with dimensions consistent with either 1 or 2 cytochrome *c* molecules going between the phospholipid bilayers. This is in contrast to the globular structure of the isooctane-soluble complexes[13]. In isooctane, cytochrome *c* binds approximately only one tenth of the amount of phospholipid bound in water. Furthermore, whilst phosphatidylcholine is not essential for complex formation in water, only in its presence may isooctane-soluble complexes be formed. Taken together these results imply a gross structural reorganisation at the isooctane–water interface during extraction of the complex. The alcohol used to facilitate extraction may assist this reorganisation.

The outstanding problems unresolved in the present work are the mode of binding of the phosphatidylcholine, and the nature of the forces stabilizing the large-molecular-weight aggregates in isooctane. The continuation of our studies using physical techniques may contribute to the solution of these problems. In addition, monolayer studies may help in the understanding of the structural rearrangement occurring at the isooctane–water interface during extraction.

ACKNOWLEDGEMENTS

The authors would like to thank Professor D. Chapman and Dr. G. G. Shipley for helpful discussions during the course of the work.

REFERENCES

1 D. CHAPMAN, V. B. KAMAT, J. DE GIER AND S. A. PENKETT, *J. Mol. Biol.*, 31 (1968) 101.
2 D. W. URRY, M. MEDNIEKS AND E. BEJNAROWICZ, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 1043.
3 D. F. H. WALLACH AND P. H. ZAHLER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1552.
4 J. LENARD AND S. J. SINGER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1828.
5 D. F. H. WALLACH AND A. GORDON, *Federation Proc.*, 27 (1968) 1263.
6 A. SCANU, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1699.
7 A. SCANU AND R. HIRZ, *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 890.
8 D. CHAPMAN, R. B. LESLIE, A. SCANU AND R. HIRZ, *Nature*, 221 (1969) 260.
9 D. E. GREEN AND S. FLEISCHER, *Biochim. Biophys. Acta*, 70 (1963) 554.
10 M. L. DAS, E. D. HAAK AND F. L. CRANE, *Biochemistry*, 4 (1965) 859.
11 M. L. DAS AND F. L. CRANE, *Biochemistry*, 3 (1964) 696.
12 D. D. ULMER, *Biochemistry*, 4 (1965) 902.
13 G. G. SHIPLEY R. B. LESLIE AND D. CHAPMAN, *Biochim. Biophys. Acta*, 173 (1969) 1.
14 G. G. SHIPLEY, R. B. LESLIE AND D. CHAPMAN, *Nature*, 222 (1969) 561.
15 J. FOLCH, *J. Biol. Chem.*, 146 (1942) 35.
16 G. ROUSER AND S. FLEISCHER, *Methods of Enzymology*, Academic Press, New York, 1967, p. 385.
17 D. PAPAHADJOPOULOS AND N. MILLER, *Biochim. Biophys. Acta*, 135 (1967) 624.
18 E. MARGOLIASH AND A. SCHEJTER, *Advan. Protein Chem.*, 21 (1966) 113.
19 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
20 O. S. CHEN, T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 23 (1956) 1756.
21 M. REICH AND W. W. WAINIO, *J. Biol. Chem.*, 236 (1961) 3058.
22 M. L. DAS, H. HIRATSUKA, J. M. MICHINIST AND F. L. CRANE, *Biochim. Biophys. Acta*, 60 (1962) 433.