

Interactions of C-reactive protein and complement with liposomes

(phosphocholine/phospholipids/galactocerebroside/membrane permeability)

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ABSTRACT Interactions between C-reactive protein (CRP) and liposomal model membranes containing phosphatidylcholine were investigated. These interactions, in the presence of human serum, resulted in consumption of each of the components of the classical complement pathway (C1-C9) and also resulted in complement-dependent damage and release of trapped glucose from certain types of liposomes. CRP-initiated lysis of liposomes was strongly dependent upon membrane lipid composition. Optimal activity occurred with positively charged liposomes containing galactosylceramide (galactocerebroside); positively charged liposomes lacking galactocerebroside released much less glucose, while negatively charged liposomes, either with or without galactocerebroside, did not release glucose at all. Glucose release was inhibited by free phosphocholine. Lesser, but significant, "background" glucose release independent of the presence of CRP also was observed with positively charged liposomes containing galactocerebroside, and this was associated with marked preferential consumption of the later-acting complement components (C3-C9). C2-deficient human serum failed to support CRP-dependent glucose release, but glucose release was observed upon reconstitution of the serum with C2. Guinea pig complement also did not support CRP-mediated glucose release, but upon addition of human C1q substantial glucose release was observed. We conclude that (i) CRP can sensitize appropriate liposomes for complement-dependent damage via the primary complement pathway starting at the level of C1q; (ii) of those studied, liposomes that are most susceptible to membrane damage contain phosphatidylcholine, have a positive charge, and contain a ceramide glycolipid; and (iii) such liposomes also are sensitive, although to a much lesser degree, to complement-dependent lysis initiated in the absence of CRP and involving consumption of terminal in excess of early acting complement components.

C-reactive protein (CRP) is an acute phase reactant (1) which shares many of the functions of antibodies, including an ability to activate the complement (C) system via the classical pathway (2-6). CRP was originally recognized by its ability to precipitate with the pneumococcal C polysaccharide, a phosphocholine-containing teichoic acid (7-9). Reactivity of CRP with C polysaccharide was attributed to a specific interaction with phosphate esters, particularly phosphocholine (2, 10, 11). This specificity led to testing of reactivity of CRP with emulsions consisting of cholesterol and either lecithin (phosphatidylcholine) or sphingomyelin (ceramide phosphocholine). Such interactions resulted in activation of the classical C pathway with depletion of hemolytic C activity in human serum (2, 3).

In recent years, well-defined artificial membranes (liposomes) have been investigated as models for membrane-associated antigen-antibody-complement-interactions (12, 13). Liposomes consist of concentric lamellae of lipid bilayer membranes. Generally liposomes are composed of phospholipids such as lecithin or sphingomyelin, but they may also have

other lipid constituents such as cholesterol, charged lipids, and glycolipids. Liposomes can be prepared with a marker such as glucose trapped in the aqueous interspaces that separate the concentric membranes. The release of such a marker can serve to quantify complement-dependent membrane damage initiated by an interaction between antibody and liposomal antigen. We report herein that interactions of CRP with certain liposomal phospholipids result both in consumption of hemolytic complement and in complement-dependent membrane damage and release of trapped glucose.

MATERIALS AND METHODS

Chemicals were purchased from the following companies: dimyristoyl lecithin and cholesterol (Calbiochem, La Jolla, CA); dicetyl phosphate and stearylamine (K and K Laboratories, Plainview, NY); beef galactocerebroside (Schwarz/Mann, Orangeburg, NY); phosphocholine chloride, calcium salt (Sigma Chemical Co., St. Louis, MO).

Previous publications should be consulted for complete details on the following: preparation of liposomes, measurement of trapped liposomal glucose, and complement-dependent release of trapped glucose (14, 15); preparation and properties of C-reactive protein (6); measurement of serum levels of CRP (16); preparation of serum depleted of CRP (6); and consumption of hemolytic complement and individual complement components (5). Human C1q was prepared by the method of Yonemasu and Stroud (17). Additional details are given in the individual figure or table legends.

RESULTS

Glucose Release in the Presence of CRP and Complement. Liposomes containing various lipids were tested for CRP-initiated complement-dependent glucose release. The most reactive liposomes consisted of a combination of dimyristoyllecithin, cholesterol, galactocerebroside or certain other ceramide lipids, and a high concentration of stearylamine to provide a strong positive charge. As shown in Table 1, liposomes lacking galactocerebroside were much less reactive, and negatively charged liposomes were not reactive at all. A more detailed report of the unusual membrane requirements for CRP-initiated complement-dependent glucose release will be presented elsewhere.

Fig. 1 demonstrates that glucose release was related to the amount of CRP present. Incubation of the maximally reactive liposomes with complement alone resulted in a relatively high baseline of glucose release (Table 1; Fig. 1). Glucose release in excess of this baseline was seen in the presence of CRP, and it occurred with a sigmoidal dose-response curve which reached

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Abbreviations: CRP, C-reactive protein; C, complement; C^{hu} and C^{gp}, human and guinea pig complement; GalCer, galactosylceramide (galactocerebroside).

Table 1. Influence of lipid composition on CRP-mediated complement damage to liposomes

CRP (μg)	Glucose release (%) from:			
	Positive plus GalCer	Positive	Negative plus GalCer	Negative
0	14	7.9	1.1	1.5
8.8	36	11	2.6	1.5
26.5	37	19	0.6	0.5

Liposomes (5 μl) consisted of dimyristoyl lecithin, cholesterol, stearylamine (for positive charge), or dicetyl phosphate (for negative charge) and galactocerebroside (GalCer), where indicated. Dimyristoyl lecithin, cholesterol, and charged lipid were in molar ratios of 2:1.5:0.66, and beef galactocerebroside was added in the amount of 150 $\mu\text{g}/\mu\text{mol}$ of lecithin. The lecithin concentration was 10 mM with respect to the aqueous dispersion of lipids. Each assay cuvette contained 500 μl of glucose assay reagent, 5.0 μl of liposomes, 100 μl of fresh human serum as a complement source, CRP (dissolved in 0.15 M NaCl), and sufficient 0.15 M NaCl to bring to 1 ml. Glucose release was measured after 30 min at room temperature (*ca* 23°).

a maximum plateau (Fig. 1). The magnitude of the plateau varied with each individual CRP preparation, but was consistent for each batch, and ranged from 30 to 60% release of trapped glucose; that used for most of the experiments presented herein gave 35–45% glucose release. Although the reasons for the differences between activities of different CRP batches are not completely understood, we have confirmed the finding of MacLeod and Avery (8) that some lipid is associated with purified CRP, and this may be one factor that could influence the reactions described. Fig. 2 demonstrates that free phosphocholine completely inhibited activity of CRP against liposomes.

A complement titration experiment is illustrated in Fig. 3. The high baseline or "background" of glucose release shown in Fig. 1 was not seen in the absence of complement (Fig. 3; see below), indicating that it did not result from simple instability of the liposomes. The degree of background release varied

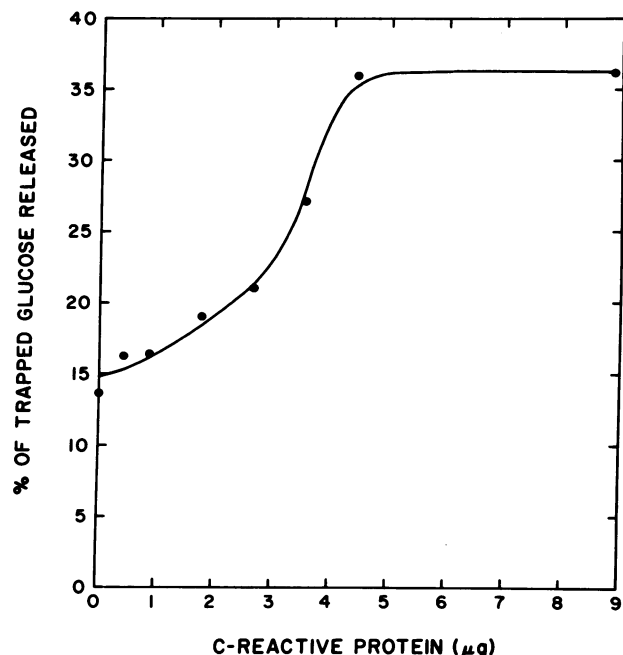


FIG. 1. Dependence of glucose release on CRP. Positive liposomes (plus GalCer) were prepared and glucose release was measured as described in the legend of Table 1.

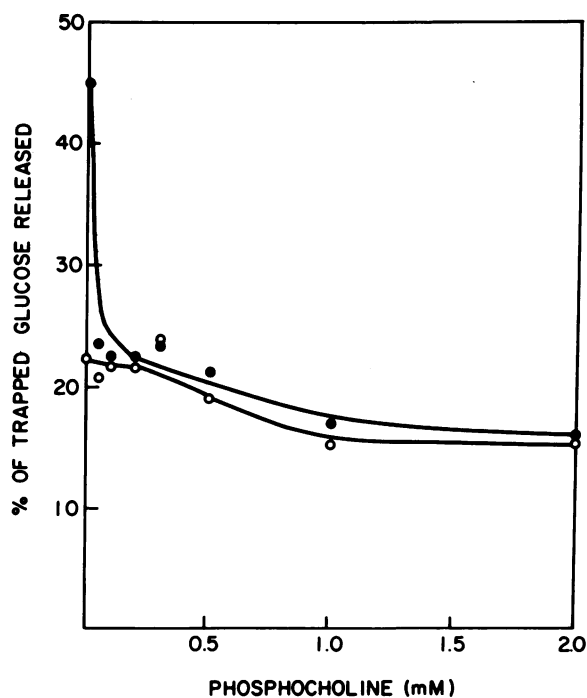


FIG. 2. Inhibition of glucose release by phosphocholine. Liposomes were incubated as described in the legend of Table 1, except that the amounts of phosphocholine shown on the abscissa were present, and 3 μg of CRP was used. This batch of CRP was more active than that illustrated in Fig. 1. ●, Human C (C^{hu}) + CRP; ○, C^{hu} alone.

widely depending on the human serum used, and separate experiments showed that it was not related to the concentration of the trace amounts of CRP present in individual serum samples. Although the mechanism underlying the background glucose release is not yet completely clear, it was slightly, but consistently, inhibited by free phosphocholine (Fig. 2).

Requirement for Complement. Glucose release due to CRP was completely blocked by C inactivation by heating or hydrazine, and by C depletion by zymosan or insoluble immune complexes (Table 2). Sera deficient in C2 did not support either CRP- or antibody-initiated glucose release; activity with CRP was fully restored, and activity with antibody was partially restored, upon addition of purified C2. In the experiment illustrated in Table 2 the background glucose release (–CRP) due to the particular batch of human serum used was very high, but

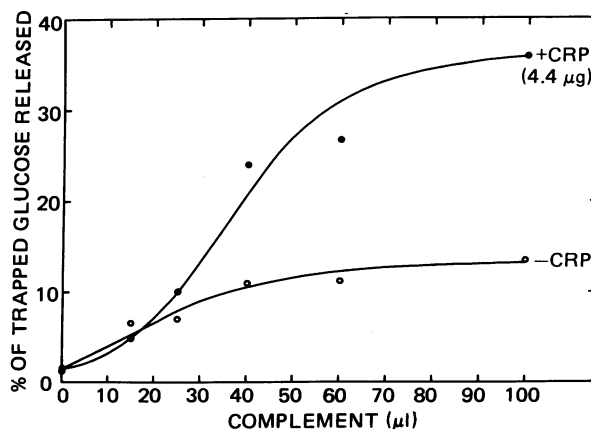


FIG. 3. Dependence of glucose release on complement. Positive liposomes (plus GalCer) were prepared and glucose release was measured as described in the legend of Table 1.

Table 2. Human complement requirement for CRP-mediated glucose release

Complement	Glucose release (%)		
	-CRP	+CRP	+Antiserum
None	4.8	4.4	<1
C ^{hu}	24	39	48
Heated C ^{hu}	8	6.1	<1
Zymosan C ^{hu}	12	8.6	<1
AHGG C ^{hu}	12	8.5	<1
Antigen-Antibody-C ^{hu}	11	7.8	<1
Hydrazine C ^{hu}	13	5.7	<1
C2-Deficient C ^{hu}	8	6.2	<1
C2-Deficient C ^{hu} + C2	11	21	6.1
C ^{gp}	14	4.7	
C ^{gp} + human C1q	13	29	

The glucose release assays were performed as described in the legend of Table 1, using 4.4 μg of CRP and positive liposomes plus GalCer. Where indicated, 15 μl of rabbit anti-galactocerebroside serum was substituted for CRP in order to test the activity of the complement. For this latter purpose the liposomes contained dimyristoyl lecithin/cholesterol/dicetyl phosphate (2:1.5:0.22) and 150 μg of GalCer per μmol of lecithin. As shown, the complement source consisted of 100 μl of normal human (hu) or guinea pig (gp) serum, C2-deficient human serum, or normal human serum treated with: heat (56°, 30 min), hydrazine (18 mM, 37°, 2 hr), zymosan (4 mg/ml, 37°, 60 min), insoluble precipitate of bovine serum albumin-anti-bovine serum albumin (0.8 mg/ml, 37°, 60 min), aggregated human gamma globulin (AHGG, 0.8 mg/ml, 37°, 60 min). AHGG was prepared by heating human immune serum globulin (Armour Pharmaceutical Co., Phoenix, AZ) at 63° for 20 min. Where shown, 250 CH₅₀ (50% hemolysis) units of human C2 (Cordis Laboratories, Miami, FL; dialyzed against 0.15 M NaCl) or 30 μg of human C1q (in Veronal-buffered saline) was also present.

it also was at least partly dependent on intact complement activity. Glucose release was not seen when guinea pig complement was substituted for human complement unless, as in the experiments of Volanakis and Kaplan involving human CRP and C polysaccharide (3), human C1q was added (Table 2).

Complement Consumption by Liposomes. The ability of liposomes to consume hemolytic C in the presence of CRP was investigated in the next series of experiments. The positively charged liposomes containing galactocerebroside were C consuming even in the absence of CRP, but consumption was greatly enhanced when CRP was added (Fig. 4); approximately

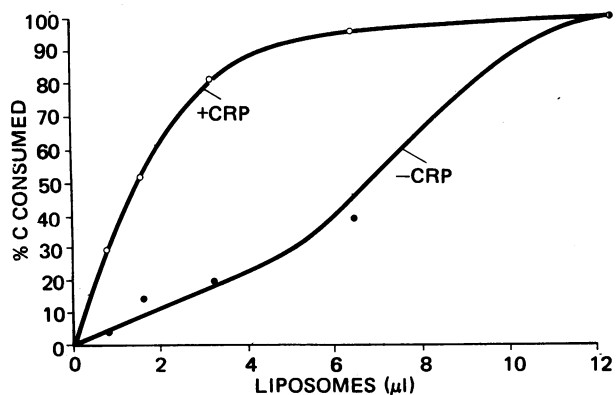


FIG. 4. Dependence of C consumption on liposome concentration. CRP-depleted serum (0.2 ml) was incubated (60 min, 30°) with various amounts of liposomes in a total volume of 1.0 ml in Veronal-buffered NaCl (0.1 M relative salt concentration) in the presence or absence of CRP (80 $\mu\text{g}/\text{ml}$). Residual hemolytic activity was determined as described elsewhere (4). Liposomes were dimyristoyl lecithin/cholesterol/stearylamine/GalCer.

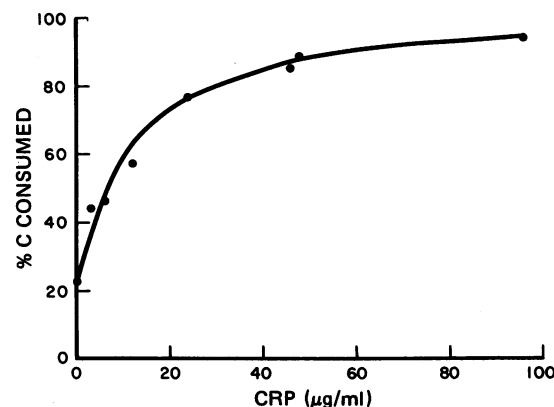


FIG. 5. Dependence of C consumption on CRP. Using 10 μl of liposomes (composition as in Fig. 4) and varying CRP, C consumption was assayed as described in the legend of Fig. 4.

5-fold larger amounts of liposomes were required for 50% C consumption in the absence of, as compared to the presence of, CRP (7.0 μl and 1.5 μl , respectively). The addition of increasing amounts of CRP to a mixture of human serum and liposomes resulted in increased amounts of C consumption (Fig. 5). Analysis of the C component depletion profile showed that the liposomes in the absence of CRP initiated substantial consumption of each of the six C3-C9 components as well as a small amount of C2; essentially no depletion of C1 or C4 was observed (Fig. 6A). By contrast, in the presence of CRP, marked consumption of C1, C4, and C2 as well as C3-C9 was observed. Indeed, the depletion of early-acting components was enhanced, while the depletion of certain terminal components seemed to be decreased, in the presence of CRP (Fig. 6B).

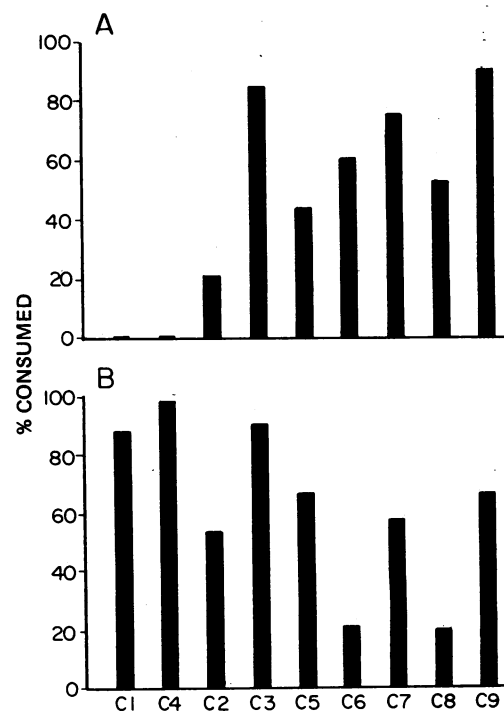


FIG. 6. C component depletion profiles induced by interaction of liposomes with human serum in the presence and absence of CRP. CRP-depleted human serum (0.2 ml) was incubated (60 min, 30°) with liposomes (100 μl) in a total volume of 1.0 ml in the absence (A) or presence (B) of CRP at 80 $\mu\text{g}/\text{ml}$. Residual hemolytic activity of each of the C1-C9 complement components was determined as described previously (5).

DISCUSSION

Our experiments demonstrate that CRP reacted with liposomes containing phosphatidylcholine, resulting in activation of the classical human complement pathway. These findings therefore confirm the demonstration by Kaplan and Volanakis (2) that phosphatidylcholine can serve as a ligand for binding of CRP, and that the interaction results in consumption of hemolytic complement. Our results also show that complement activation caused by CRP can result in liposomal membrane damage and release of trapped marker. Glucose release was dependent on CRP concentration, and the CRP was inhibited by free phosphocholine. These reactions are analogous, therefore, to complement-dependent membrane damage initiated by antibody binding (13). In addition to other complement components, C2 and C8 were required for glucose release in the presence of antibodies (18); we have demonstrated that complement-dependent glucose release in the presence of CRP apparently has a similar requirement for C2 (Table 2) and all of the classical pathway hemolytic complement components are consumed (Fig. 6B). Furthermore, only very small quantities of CRP-initiated glucose release occurred when guinea pig serum was substituted for human serum as a complement source, but glucose release mediated by guinea pig serum was stimulated by human C1q (Table 2). This finding is consistent with previous observations that guinea pig complement was not activated as efficiently as human complement in the presence of human CRP unless human C1q was present (3, 6). We conclude, therefore, that CRP-initiated glucose release from liposomes probably requires activation of the classical complement pathway.

The mechanism of complement-dependent glucose release mediated by CRP apparently is different from the mechanism of release caused by certain other initiators. In the presence of CRP, complement-dependent membrane damage occurred only with positively charged liposomes and was greatly enhanced by galactosylceramide in the liposomes (Table 1). It should be pointed out that the data of Table 1 did not distinguish whether diminished glucose release was due to decreased binding of CRP or to decreased complement fixation. Separate adsorption experiments demonstrated that CRP was bound, although to a lesser extent, both to negative liposomes and to positive liposomes lacking galactosylceramide. Even under conditions when CRP is maximally bound, however, variations of membrane lipid composition can strongly influence consumption of hemolytic complement by liposomes. The influence of membrane composition on CRP-mediated membrane damage will be explored in more detail in a later publication. In contrast to the CRP system, complement-dependent glucose release initiated either by antibodies (13) or by "reactive lysis" (19) was independent of either liposomal charge or ceramide lipids. Galactosylceramide originally was included in the present experiments because antibodies against this substance could be used, if necessary, as a "positive control" for complement-mediated glucose release. It is of interest that ceramide in lipid membranes also had an enhancing effect on cholesterol-dependent complement activation (15) and on the activity of phospholipase C (20). Ceramide might have the ability to modulate activities of certain substances at the surface of the lipid bilayer membrane.

In the course of these studies we found that in the absence of CRP a large volume of positively charged liposomes containing galactocerebroside consumed all of the classical complement components, starting at C2 or C3 (Fig. 6A). This consumption presumably was responsible for the high background of glucose release observed with normal, but not with

C2-deficient, human complement (Table 2). The background glucose release differed with different complement donors, and this suggests the possibility that another circulating factor, independent of CRP, also reacted with the liposomal phospholipids and caused complement activation. This concept is supported by experiments which showed that the background was partially inhibited by high concentrations of phosphocholine (Fig. 2), but persisted even after removal of CRP on an affinity column (not shown).

We conclude that liposomal membranes may be useful as models to investigate the properties of membrane-associated complement activation by CRP. Previous studies have shown that certain complement components starting at C5 can bind to protein-free liposomes, resulting in membrane damage and marker release ("reactive lysis") (19). Presumably, the substrates for intermediate complement binding (e.g., C5b, C567) and for terminal transformation (after reaction of C8 or C9) are phospholipids, and therefore correspond to the same molecules that serve as receptors for CRP. It is evident, therefore, that CRP might have a regulatory influence on the activation of reactive lysis components, and on end-stage membrane damage caused by complement activation.

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- Hedlund, P. (1961) "Clinical and experimental studies on C-reactive protein (acute phase protein)," *Acta Med. Scand. Suppl.* **361**, 1-71.
- Kaplan, M. H. & Volanakis, J. E. (1974) "Interactions of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin, and sphingomyelin," *J. Immunol.* **112**, 2135-2147.
- Volanakis, J. E. & Kaplan, M. H. (1974) "Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: Requirement for human C1q," *J. Immunol.* **113**, 9-17.
- Siegel, J., Rent, R. & Gewurz, H. (1974) "Interactions of C-reactive protein with the complement system. I. Protamine-induced consumption of complement in acute phase sera," *J. Exp. Med.* **140**, 631-647.
- Siegel, J., Osmand, A. P., Wilson, M. F. & Gewurz, H. (1975) "Interactions of C-reactive protein with the complement system. II. C-reactive protein-mediated consumption of complement by poly-L-lysine polymers and other polycations," *J. Exp. Med.* **142**, 709-721.
- Osmand, A. P., Mortensen, R. F., Siegel, J. & Gewurz, H. (1975) "Interactions of C-reactive protein with the complement system. III. Complement-dependent passive hemolysis initiated by CRP," *J. Exp. Med.* **142**, 1065-1077.
- Tillett, W. S. & Francis, T., Jr. (1930) "Serological reactions in pneumonia with a nonprotein somatic fraction of pneumococcus," *J. Exp. Med.* **52**, 561-571.
- MacLeod, C. M. & Avery, O. T. (1941) "The occurrence during acute infections of a protein not normally present in the blood. III. Immunological properties of the C-reactive protein and its differentiation from normal blood proteins," *J. Exp. Med.* **73**, 191-200.
- Brundish, D. E. & Baddiley, J. (1968) "Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate," *Biochem. J.* **110**, 573-582.
- Gotschlich, E. C. & Edelman, G. M. (1967) "Binding properties and specificity of C-reactive protein," *Proc. Natl. Acad. Sci. USA* **57**, 706-712.
- Volanakis, J. E. & Kaplan, M. H. (1971) "Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide," *Proc. Soc. Exp. Biol. Med.* **136**, 612-614.
- Kinsky, S. C. (1972) "Antibody-complement interaction with lipid

- model membranes," *Biochim. Biophys. Acta* **265**, 1-23.
13. Alving, C. R. (1977) "Immune reactions of lipids and lipid model membranes," in *The Antigens*, ed. Sela, M. (Academic Press, New York), Vol. 4, pp. 1-72.
 14. Kinsky, S. C. (1974) "Preparation of liposomes and a spectrophotometric assay for release of trapped glucose marker," in *Methods in Enzymology*, eds. Fleischer, S. & Packer, L. (Academic Press, New York), Vol. 32, pp. 501-513.
 15. Alving, C. R., Richards, R. L. & Guirguis, A. A. (1977) "Cholesterol-dependent human complement activation resulting in damage to liposomal model membranes," *J. Immunol.* **118**, 342-347.
 16. Claus, D. R., Osmand, A. P. & Gewurz, H. (1976) "Radioimmunoassay of human C-reactive protein and levels in normal sera," *J. Lab. Clin. Med.* **87**, 120-128.
 17. Yonemasu, K. & Stroud, R. M. (1971) "C1q: Rapid purification method for preparation of monospecific antisera and for biochemical studies," *J. Immunol.* **106**, 304-313.
 18. Haxby, J. A., Götze, O., Müller-Eberhard, H. J. & Kinsky, S. C. (1969) "Release of trapped marker from liposomes by the action of purified complement components," *Proc. Natl. Acad. Sci. USA* **64**, 290-295.
 19. Lachmann, P. H., Munn, E. A. & Weissmann, G. (1970) "Complement-mediated lysis of liposomes produced by the reactive lysis procedure," *Immunology* **19**, 983-986.
 20. Demel, R. A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofs, B. & van Deenen, L.L.M. (1975) "Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers," *Biochim. Biophys. Acta* **406**, 97-107.