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Antibody Binding and Complement Fixation by a Liposomal Model Membrane*

Carl R. Alving, Stephen C. Kinsky, James A. Haxby, and Constance B. Kinsky

ABSTRACT: Previous studies have shown that liposomal model membranes, prepared with fractions obtained from sheep erythrocytes, release trapped glucose in the presence of rabbit antisheep erythrocyte serum and unheated guinea pig serum. The present experiments demonstrate that: (1) these liposomes can bind (neutralize) antibodies in the antiserum, which are responsible for sheep erythrocyte hemolysis, but only when the appropriate antigen has been incorporated into the liposomal structure, and (2) liposomes, which contain antigen, are able to fix guinea pig complement but only in the presence of antiserum.

These observations further support the feasibility of using liposomes as an artificial membrane system for studying certain aspects of complement mechanism.

In recent years, the major emphasis of studies dealing with immune lytic mechanisms has been directed toward isolation of complement components and establishing the order in which they react to produce a structural defect in cell membranes. Although notable progress along these lines has been made in numerous laboratories (see review by Muller-Eberhard, 1968), it seems apparent that explanation of complement-induced lysis on a molecular basis is also contingent on the identification of the membrane constituents which participate in this process and the manner in which these are organized in cell membranes before and after lysis.

The possibility that artificial membranes might provide some useful information on this point prompted us to attempt construction of a liposomal model membrane from sheep erythrocyte lipids that would respond to antibody and complement (Haxby *et al.*, 1968). The choice of this artificial membrane system, and starting material, was dictated by several considerations. Liposomes rather than lipid monolayers or thin lipid (bilayer or "secondary black") films were employed because their preparation does not require any unique equipment, and permeability alterations can be conveniently determined by a simple spectrophotometric procedure. This method utilizes glucose

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as the trapped marker and its release from the liposomes is measured by the change in absorbance at 340 mμ which occurs upon the reduction of TPN^+ in the presence of hexokinase, glucose 6-phosphate dehydrogenase, and the requisite cofactors (Kinsky *et al.*, 1968; Demel *et al.*, 1968; Haxby *et al.*, 1968). Another reason for studying liposomes is that they, unlike mono- or bilayer films, can be readily examined in the electron microscope with negative staining techniques. This is a particularly advantageous property because of the numerous studies which have shown that membranes, obtained from various mammalian and bacterial cells that have undergone immune lysis, contain characteristic lesions or "pits" (Borsos *et al.*, 1964; Humphrey and Dourmashkin, 1965; Rosse *et al.*, 1966, Bladen *et al.*, 1966, 1967; Mergenhagen *et al.*, 1968). These pits were initially found in membranes from sheep erythrocytes that had been lysed by anti-Forssman antibodies in the presence of complement. Because most of the information on complement mechanism has come from the study of sheep erythrocyte lysis by rabbit antiserum, we decided to use these cells for the isolation of material with which to prepare liposomes. In this regard, it should also be noted that Forssman antigen activity is associated with a glycolipid fraction (Papirmeister and Mallette, 1955; Yamakawa *et al.*, 1960; Rapp and Borsos, 1966) whose amphipathic properties might possibly favor the proper insertion of the antigen into the lipid lamellae of the liposomes.

The feasibility of the approach outlined above was indicated by the experiments described in the first paper of this series (Haxby *et al.*, 1968). We were able to obtain two fractions from sheep erythrocyte membranes: fraction IIa (chloroform soluble) which contains the phospholipids, cholesterol, and some of the antigen activity present in the membrane, and fraction IIb (methanol–water soluble) which contains antigen but not the major lipids. From fraction IIa, it was possible to prepare liposomes with trapped glucose and this glucose was released upon incubation with rabbit antisheep erythrocyte serum and guinea pig serum. We were not able to prepare liposomes from fraction IIb alone; however, when this fraction was incorporated into an artificial lipid mixture containing sphingomyelin, cholesterol, and dicetyl phosphate, liposomes were formed which also released marker in the presence of antiserum and guinea pig serum. Glucose release did not occur if serum from nonimmunized rabbits was employed or if the guinea pig serum was heated for 30 min at 56°. These observations suggested that release of marker occurs as a consequence of a genuine "immune response" of the liposomal model membrane requiring the participation of both antibodies and complement. The experiments described below provide further support for this conclusion by showing that preincubation with these liposomes can inhibit immune hemolysis by binding (neutralizing) the responsible antibodies and by fixing complement.

Experimental Procedures

The materials and assay methods used in the current investigation were the same as those employed in the experiments previously published (Haxby *et al.*, 1968). This paper should be consulted for details regarding: (a) sources of the various chemicals, enzymes, coenzymes, and immunologic reagents; (b) isolation of fractions IIa and IIb from sheep erythrocyte membranes; (c) preparation of liposomes with these fractions; and (d) spectrophotometric method for following glucose release.

The methods described by Mayer (1961) were used to: (a) make Veronal-buffered saline with and without 0.1% gelatin; (b) prepare a stock suspension of washed sheep erythrocytes containing 19^9 cells/ml; (c) sensitize sheep erythrocytes with rabbit antiserum; and (d) titrate complement.

Antibody Binding. Binding of antibodies to liposomes was examined by comparing the ability of rabbit antiserum, preincubated in the presence and absence of liposomes, to sensitize sheep erythrocytes. Liposomes (15 μl, equivalent to 0.15 μmole of phospholipid) were added to Corex tubes (15 × 100 mm) containing 2.91 ml of Veronal–saline–gelatin and 75 μl of appropriately diluted samples of rabbit antiserum such that the final dilution ranged from 1:400 to 1:6400. Control tubes were identical with the above except that the liposomes were omitted. After incubation for 20 min at room temperature (*ca.* 22°), the liposomes were removed by centrifugation (27,000*g*) for 15 min at 2°. Sheep erythrocytes were sensitized by mixing 1 ml of the supernatant solution from each tube with 1 ml of the stock erythrocyte suspension. Each sensitized erythrocyte suspension (0.2 ml) was added to chilled tubes (8 × 75 mm) followed by 1.1 ml of Veronal–saline–gelatin and 0.2 ml of a 1:125 dilution of guinea pig serum as the source of complement; the latter contained 202 $C'H_{50}$ units/ml undiluted serum. After 1 hr at 37°, any intact cells were removed by centrifugation and the extent of hemolysis was determined from the absorbance of the supernatant solution at 541 mμ.

A modification of this procedure was used to determine if liposomes prepared from fraction IIa bound antibodies which were necessary to release glucose from IIb liposomes and, conversely, if IIb liposomes bound antibodies which were required for the release of marker from IIa liposomes.[1] In this case, tubes containing 0.23 ml of a 1:100 dilution of rabbit antiserum, varying amounts of the appropriate liposomes, and sufficient water to give a final volume of 0.35 ml were incubated for 30 min at 37°. The liposomes were then removed by centrifugation and 100 μl of the supernatant solution was used in the spectrophotometric assay (Haxby *et al.*, 1968). Additional details are

[1] For the sake of convenience, we will occasionally refer to liposomes prepared from fraction IIa as "IIa liposomes" and those prepared from a lipid mixture containing sphingomyelin, cholesterol, and dicetyl phosphate, *plus* fraction IIb, as "IIb liposomes." Liposomes made from the lipid mixture only (*i.e.*, without incorporation of fraction IIb) will be called "sphingomyelin–cholesterol" liposomes. The molar ratios of the lipids, and the amount of fraction IIb used in the preparation of these liposomes, are indicated in the appropriate figure legends.

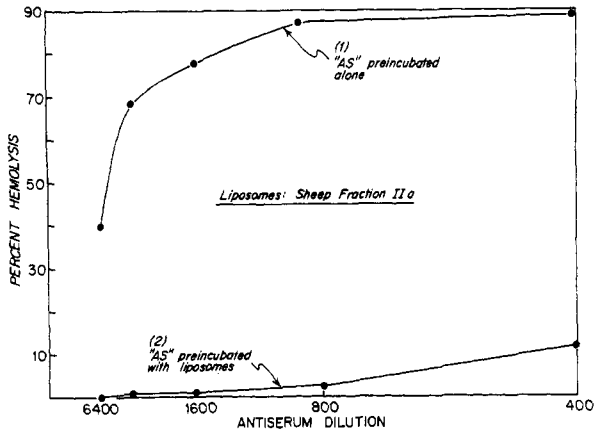

FIGURE 1: Neutralization (binding) of antibodies by IIa liposomes. Binding of hemolytic antibodies in rabbit anti-sheep erythrocyte serum ("AS") by IIa liposomes was determined by the method described in Experimental Procedures. The abscissa indicates the final dilution of antiserum present during preincubation. The per cent hemolysis values have been corrected for any spontaneous lysis (approximately 2%) occurring in the absence of complement.

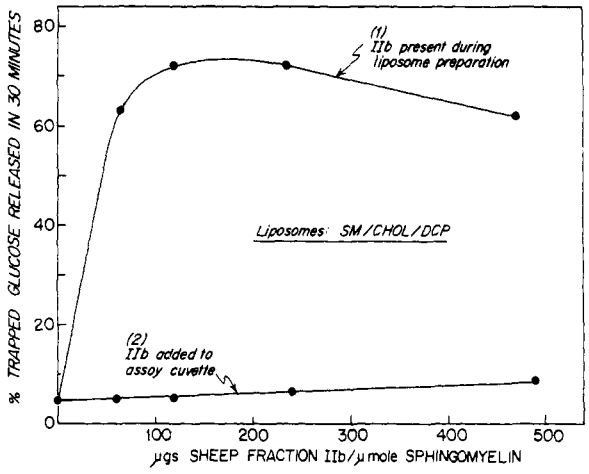

FIGURE 2: Requirement for sheep fraction IIb incorporation into sphingomyelin–cholesterol liposomes. In the experiment shown in curve 1, liposomes were prepared from a lipid mixture containing sphingomyelin (SM), cholesterol (CHOL), and dicetyl phosphate (DCP) plus various amounts of sheep fraction IIb. The molar ratios of SM, CHOL, and DCP were 2:1.5:0.41, respectively, and the abscissa indicates the micrograms of fraction IIb per micromole of sphingomyelin in the liposome preparations. Glucose release from these liposomes was determined by the spectrophotometric method of Haxby *et al.* (1968) in the presence of 11.1 μl of a 1:10 dilution of rabbit antiserum and 57.3 μl of guinea pig serum (containing 7.6 $C'H_{50}$ units of complement). In the experiment shown in curve 2, glucose release was determined under identical conditions from liposomes prepared as above with SM, CHOL, and DCP but without fraction IIb; fraction IIb was added to the assay cuvets to give the ratios shown on the abscissa. See text for additional details.

presented in the legends to Figures 4 and 5.

Complement Fixation. The method employed to determine if liposomes can fix complement was as follows. Liposomes (15 μl) were added to Corex tubes containing 30 μl of a 1:10 dilution of rabbit antiserum, varying quantities of guinea pig serum (not exceeding 1 $C'H_{50}$ unit), and sufficient Veronal–saline–gelatin to give a final volume of 3 ml. Control tubes were identical with the above except that either liposomes or antiserum were omitted. After incubation for 30 min at 37°, the liposomes were removed by centrifugation. A 1.3-ml aliquot of each supernatant solution was transferred to 8 × 75 mm tubes, followed by the addition of 0.2 ml of a suspension of sheep erythrocytes which had been previously sensitized with rabbit antiserum. The latter was prepared by mixing equal volumes of the stock erythrocyte suspension with a 1:600 dilution of the antiserum. The extent of hemolysis was determined after incubation for 1 hr at 37° as described above for the "antibody binding" experiments.

Results

Antibody Binding by Liposomes. The ability of rabbit antiserum to sensitize sheep erythrocytes was markedly reduced by prior incubation of the antiserum with IIa liposomes. This is shown by comparison of curve 1 with curve 2 in Figure 1. These results suggest that liposomes can bind, and thereby neutralize, antibodies which are responsible for sheep erythrocyte hemolysis, but do not *per se* eliminate the possibility of a non-specific interaction.

The above experiment was therefore repeated with IIb liposomes to determine whether antibody neutralization by liposomes was indeed immunologically specific, *i.e.*, involved binding of antibody to antigen. As noted earlier, liposomes prepared from an artificial lipid mixture containing sphingomyelin, cholesterol, and dicetyl phosphate, *plus* fraction IIb, release glucose when incubated with antiserum and guinea pig serum. In contrast, liposomes prepared from the lipid mixture alone do not lose any significant amount of marker under these conditions (Haxby *et al.*, 1968). For the purposes of the present investigation, it is important to emphasize that fraction IIb does not confer "immune sensitivity" to these liposomes if it is added after the liposomes have been formed. As illustrated in Figure 2, fraction IIb must be present during liposome preparation indicating that the responsible antigenic material is actually incorporated into the liposomal membrane.

Figure 3 shows that rabbit antiserum preincubated with liposomes lacking IIb (curve 2) had the same capacity to sensitize sheep erythrocytes as antiserum preincubated in the absence of liposomes (curve 1). However, when fraction IIb was incorporated into the liposomes, the sensitizing ability of the antiserum was significantly reduced after preincubation with the liposomes (curve 3). These results thus demonstrate that interaction of antibody with liposomes requires the presence of antigen in the liposomal structure.

Comparison of IIa and IIb Liposomes. The contention that the immune response of these liposomes involved interaction with anti-Forssman antibodies was based on

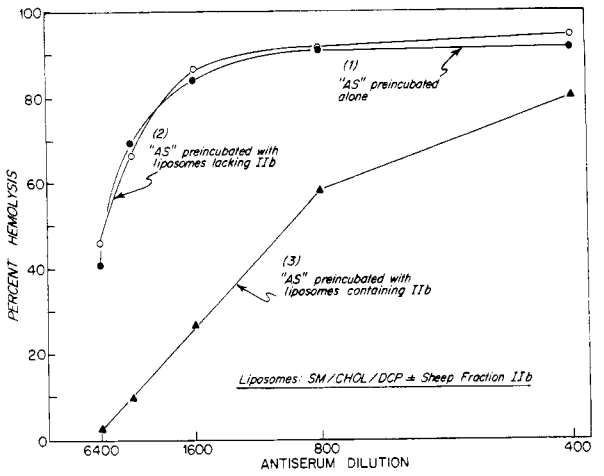

FIGURE 3: Neutralization (binding) of antibodies by sphingomyelin–cholesterol liposomes prepared with and without sheep fraction IIb. Binding of hemolytic antibodies in rabbit antisheep erythrocyte serum ("AS") by different liposome preparations was determined by the method described in Experimental Procedures. The abscissa indicates the final dilution of antiserum present during preincubation. In the experiment shown in curve 2, liposomes were made from a lipid mixture containing sphingomyelin (SM), cholesterol (CHOL), and dicetyl phosphate (DCP) in molar ratios of 2:1.5:0.22, respectively. An identical lipid mixture was used to prepare liposomes for the experiment shown in curve 3 except that sufficient sheep fraction IIb was added to give a ratio of 155 μg of fraction IIb/μmole of sphingomyelin. It should be noted that, at this ratio, fraction IIb conferred maximum immune sensitivity to the sphingomyelin–cholesterol liposomes (see Figure 2). The per cent hemolysis values have been corrected for any spontaneous lysis (approximately 2%) occurring in the absence of complement.

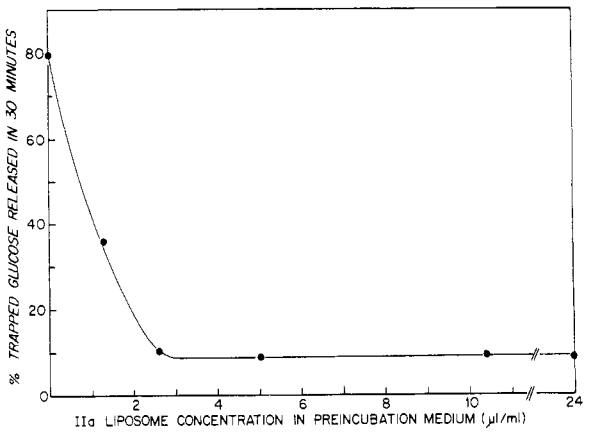

FIGURE 4: Effect of preincubation with IIa liposomes on the ability of rabbit antisheep erythrocyte serum ("AS") to cause glucose release from IIb liposomes. Antiserum was preincubated with varying amounts of IIa liposomes (as indicated on the abscissa) under the conditions described in Experimental Procedures. In this experiment, the IIa liposomes were swollen with an isotonic saline solution (0.075 M KCl–0.075 M NaCl) instead of glucose. After removal of the IIa liposomes by centrifugation, 100 μl of the superantant solution was employed in the spectrophotometric assay (Haxby *et al.*, 1968) to determine the amount of glucose released from IIb liposomes in the presence of 39.9 μl of guinea pig serum (containing 8.1 $C'H_{50}$ units of complement). The IIb liposomes were prepared from a lipid mixture containing sphingomyelin, cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.22, respectively, and sufficient sheep fraction IIb to give a ratio of 233 μg of fraction IIb/μmole of sphingomyelin.

a previous experiment showing that liposomes prepared from beef erythrocytes (which lack Forssman antigen(s)) did not release glucose when incubated with sheep hemolysin and complement (Haxby *et al.*, 1968). However, the sheep erythrocyte membrane may contain a number of different antigens which are not present in the beef cell, and, on the basis of the available data, we are not justified in concluding that the response of the liposomes is due solely to the "Forssman system." Indeed, the following observations provide some evidence that a heterogeneous group of antigens may be involved.

Figure 4 shows that preincubation of rabbit antiserum with low concentrations of IIa liposomes (*ca.* 3 μl of liposomes preparation/ml) resulted in almost a complete loss of the ability to cause glucose release from IIb liposomes. However, as illustrated in Figure 5, the antiserum retained nearly 50% of its ability to induce marker loss from IIa liposomes after preincubation with very high concentrations of IIb liposomes (*ca.* 75 μl of liposome preparation/ml). These results suggest that fraction IIb does not contain all of the antigens present in fraction IIa and, accordingly, lacks some of the antigens originally present in the sheep erythrocyte membrane. Comparison of the experiments described in Figures 1 and 3 leads to a similar

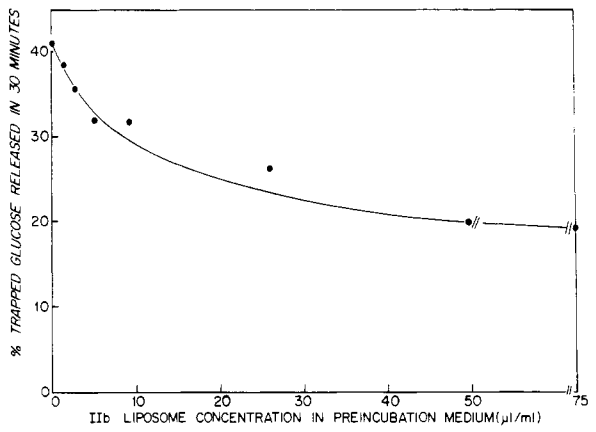

FIGURE 5: Effect of preincubation with IIb liposomes on the ability of rabbit antisheep erythrocyte serum ("AS") to cause glucose release from IIa liposomes. Procedure was similar to that described in the legend to Figure 4 except that, in this experiment, the IIb liposomes were swollen with the isotonic saline solution and the IIa liposomes contained the glucose marker.

conclusion and also suggests that fraction IIa, in contrast to fraction IIb, does possess all of the antigenic specificity of the erythrocyte. Thus, a 1:800 dilution of rabbit antiserum lost essentially all of its sensitizing ability when preincubated with IIa liposomes (Figure 1)

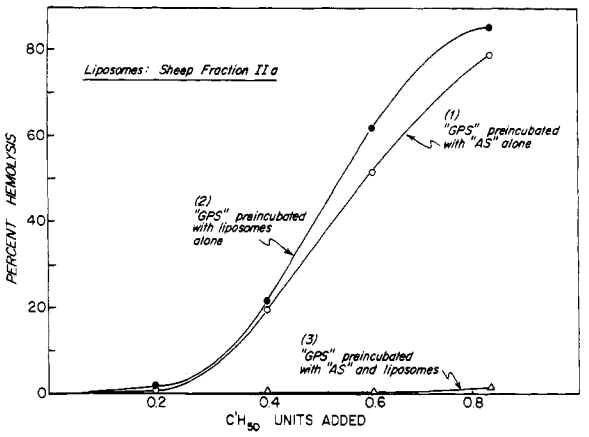

FIGURE 6: Complement fixation by IIa liposomes. Fixation of complement in guinea pig serum ("GPS") was determined by the method described in Experimental Procedures. The abscissa indicates the $C'H_{50}$ units of complement initially present during preincubation. The per cent hemolysis values have been corrected for any spontaneous lysis (approximately 6%) occurring in the absence of complement.

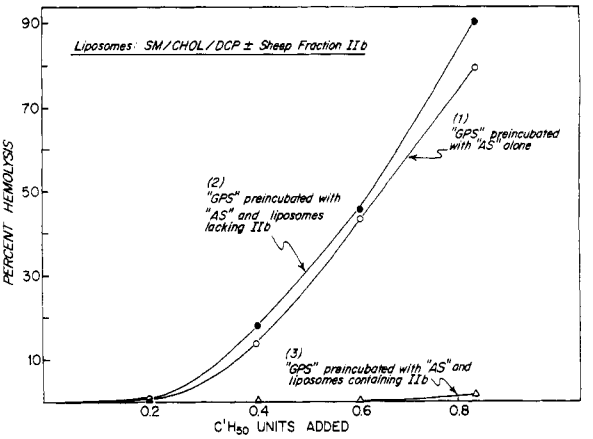

FIGURE 7: Complement fixation by sphingomyelin–cholesterol liposomes prepared with and without sheep fraction IIb. Fixation of complement in guinea pig serum (GPS) was determined by the method described in Experimental Procedures. The abscissa indicates the $C'H_{50}$ units of complement initially present during preincubation. In the experiment shown in curve 2, liposomes were made from a lipid mixture containing sphingomyelin (SM), cholesterol (CHOL), and dicetyl phosphate (DCP) in molar ratios of 2:1.5:0.22, respectively. An identical lipid mixture was used to prepare liposomes for the experiment shown in curve 3 except that sufficient sheep fraction IIb was added to give a ratio of 155 μg of fraction IIb/μmole of sphingomyelin. The per cent hemolysis values have been corrected for any spontaneous lysis (approximately 1%) occurring in the absence of complement.

whereas the same dilution of antiserum, when preincubated with IIb liposomes under identical conditions, still produced a significant sensitization of erythrocytes (Figure 3).

Complement Fixation by Liposomes. Earlier experiments have shown that the ability of guinea pig serum to stimulate glucose release was completely destroyed by heating (56° for 30 min) suggesting the participation of complement in this process (Haxby *et al.*, 1968). This conclusion is also supported by the following observations which indicate that complement is fixed under the conditions necessary to obtain release of marker from the liposomes.

As shown in Figure 6 (curve 3), no hemolysis was observed, over the range of $C'H_{50}$ units added, when guinea pig serum was preincubated with rabbit antiserum and IIa liposomes before the addition of sensitized sheep erythrocytes. However, hemolysis did occur when guinea pig serum was incubated with liposomes in the absence of antiserum and the lytic curve 2 was identical with the control curve 1 obtained by preincubating guinea pig serum with antiserum only. Thus, complement fixation by IIa liposomes requires the presence of antiserum.

Analogous experiments employing IIb liposomes further indicate that complement fixation requires the presence of antibodies directed against some antigen(s) in the liposomal membrane. The previous experiment described in Figure 3 has demonstrated that sphingomyelin–cholesterol liposomes do not bind antibodies. These liposomes also do not fix complement when preincubated with rabbit antiserum. This is illustrated by comparison of curve 2 in Figure 7 with the control curve 1 obtained by preincubating guinea pig serum with antiserum in the absence of liposomes. However, when fraction IIb was incorporated into the liposomal structure, preincubation in the presence of antiserum abolished the hemolytic activity of guinea pig serum (curve 3), indicating complete fixation of complement under these conditions.

Discussion

It is generally accepted that, in most cases of immune lysis, antibody must first be bound to antigen in the cell membrane to provide the requisite sties for the subsequent attachment of complement components (see review by Muller-Eberhard, 1968). The experiments described in the present paper indicate that liposomes, prepared with fractions obtained from sheep erythrocyte membranes, also fulfill this condition. We have been able to show that: (1) liposomes can bind (neutralize) hemolytic antibodies in rabbit antisheep erythrocyte serum but only when the appropriate antigen has been incorporated into the liposomal structure, and (2) liposomes, which contain antigen, are able to fix complement but only in the presence of antiserum.

Binding of antibody and fixation of complement occur under conditions which, as shown in the preceding paper (Haxby *et al.*, 1968), produce a significant release (*ca.* 50–70%) of trapped glucose from the liposomes. Thus, the available evidence suggests that these liposomes behave as an artificial membrane system which can mimic, to a remarkable degree, the immune response of natural membranes (such as found in sheep erythrocytes). Our results are at least consistent with the view that the region in the cell membrane, where complement is effective, may have lipids arranged in the bilayer configuration as has been

demonstrated for the structure of liposomes (Bangham *et al.*, 1965).

However, as emphasized previously (Haxby *et al.*, 1968), the opportunity afforded by liposomes to study the molecular basis of complement action cannot be fully exploited until it is known which of the components of complement are required to obtain glucose release. Complement is a complex system which requires the sequential reaction of nine recognized components to produce lysis of erythrocytes (Nelson *et al.*, 1966; Muller-Eberhard, 1968). Experiments are now in progress to determine whether all the components necessary for immune lysis are also essential to obtain loss of marker from liposomes. Several immune phenomena (such as immune conglutination, adherence, opsonization, anaphylatoxin production, etc.) also involve the participation of complement, but not all nine components, and the possibility that the response of liposomes belongs in this category must still be considered.[2]

[2] Since submission of the paper, experiments using purified human complement components have been performed at the Scripps Clinic and Research Foundation, La Jolla, Calif., in collaboration with Drs. O. Goetze and H. J. Muller-Eberhard. These studies (manuscript in preparation) indicate that glucose release from IIa liposomes is absolutely dependent upon the presence of C'2 and C'8, and is stimulated by C'9. It thus appears that all of the complement components required for maximum rates of hemolysis are also necessary for maximum marker release from the liposomal membrane. Also, pure Forssman hapten (GalNAc-Gal-Gal-Glc-ceramide), kindly provided by Professor T. Yamakawa and Dr. S. Handa, Tokyo, can substitute for fraction IIb in the experiments described in this paper, and can confer immune sensitivity to liposomes prepared with natural and synthetic phospholipids (manuscript in preparation).

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