Complement-Dependent Damage to Liposomes Prepared from Pure Lipids and Forssman Hapten*

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ABSTRACT: Previous studies have shown that liposomes prepared from the chloroform-soluble fraction (fraction IIa) of sheep erythrocyte membranes release trapped glucose marker when incubated with both rabbit antisheep erythrocyte serum (as a source of antibodies) and guinea pig serum (as a source of complement). In the present investigation, liposomes were prepared from the corresponding IIa fraction obtained from beef erythrocyte membranes (which lack Forssman antigen), and from lipid mixtures containing sphingomyelin (or lecithin), cholesterol, and dicetyl phosphate (or stearylamine). These liposomes do not release glucose when incubated with the rabbit antiserum and guinea pig serum. However, the liposomes were rendered immunologically responsive when pure Forssman hapten, isolated from sheep erythrocytes, was added to beef fraction IIa or the lipid mixtures.

The extent, and rate, of marker loss from Forssman-sensitized liposomes was dependent upon the amount of hapten incorporated in the lipid mixture used for generation of the liposomes, and upon the concentration of antiserum and complement present during assay. The extent of glucose release was essentially the same from liposomes prepared with either sphingomyelin or lecithin, and was not affected by the charge on the liposomal membrane. Antiserum absorption experiments show that sheep IIa liposomes can bind all of the antibodies required for glucose release from sphingomyelin–cholesterol liposomes which have been sensitized with the Forssman hapten, whereas the latter liposomes can bind only some of the antibodies which induce marker loss from sheep IIa liposomes. In this regard, the Forssman hapten resembles the antigenic material in the methanol–water-soluble fraction (fraction IIb) isolated from sheep erythrocytes which, as reported previously, also has the capacity to confer immune sensitivity to liposomes prepared with artificial lipid mixtures. This and related earlier investigations raise several possibilities regarding the mechanism of immune cytolysis in view of the marked parallelism between the response of the liposomal membrane and natural membranes to antibody and complement. The available data suggest that lipids alone (perhaps in bilayer configuration) may serve as "substrate" for complement, and that cell membranes may not contain any unique and specific endogenous receptor sites (protein and/or carbohydrate) for components of the complement sequence.

Previous papers have described the preparation of liposomes from the chloroform-soluble fraction of sheep erythrocyte membranes (Haxby *et al.*, 1968; Alving *et al.*, 1969). These liposomes were studied because trapped glucose marker was released in the presence of rabbit antisheep erythrocyte serum and guinea pig serum. Both sera were required to obtain this effect and glucose release did not occur when normal rabbit serum was substituted for the immune serum, or when the guinea pig serum was heated for 30 min at 56° (conditions usually employed to inactivate complement). The kinetics of glucose release and the response of the liposomes to increasing amounts of rabbit antiserum and guinea pig serum mimicked the behavior of sheep erythrocytes undergoing hemolysis in the presence of antibody and complement. A relationship between these phenomena was also indicated by the finding that liposomes can bind the antibodies in the rabbit antiserum which are responsible for sheep erythrocyte hemolysis. Antibody neutralization required the prior incorporation of antigen into the liposomal structure and these liposomes were able to fix complement, but only in the presence of antiserum.

The preceding observations suggested that these liposomes may serve as a model membrane to investigate the molecular basis of immune lysis. However, as noted in the earlier papers, at least two important questions must be answered before such efforts are undertaken. The first concerns the nature and number of complement components which are necessary to obtain release of marker in the presence of antibody. Immune lysis generally involves the participation of nine components which react in sequence, *i.e.*, components 1, 4, 2, 3, 5, 6, 7, 8, and 9. Recent experiments, using purified human components, have shown that glucose release is absolutely dependent upon the presence of components 2 and 8, and is stimulated by component 9 (J. A. Haxby, O. Götze, S. C. Kinsky, and H. J. Müller-Eberhard, manuscript in preparation). It thus appears that all of the complement components required for maximum rates of cytolysis are also necessary for maximum marker release from the liposomes.

In many of these experiments, the liposomes were generated from fractions which did not contain detectable protein (Haxby *et al.*, 1968). This raises the second question: What is the identity of the antigen(s) that confers immune sensitivity to the liposomes? In this context, "immune sensitivity" or "responsiveness" is defined as the ability of appropriate

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antibodies to produce complement-dependent damage to the model membrane, *i.e.*, permeability alterations detected as the release of glucose. Indirect evidence suggests that Forssman antigen[1] may be involved (Haxby *et al.*, 1968; Alving *et al.*, 1969). The latter is an amphipathic compound for which the following structure has been proposed (Makita *et al.*, 1966; T. Yamakawa and S. Handa, personal communication):

N-Ac-Gal(α1→3)Gal(β1→3)Gal(β1→4)-Glc-1-ceramide

Through the generosity of Professor T. Yamakawa and Dr. S. Handa of the Faculty of Medicine, University of Tokyo, we were able to obtain some pure Forssman hapten,[1] isolated from sheep erythrocytes, sufficient for the experiments reported in this paper. These indicate that the response of the lipsomes does involve the "Forssman system." Perhaps even more significant, the results demonstrate that it is possible to prepare a membrane, which can serve as a "substrate" for complement, exclusively from lipids of known structure.

Experimental Procedures

The materials used in this investigation were obtained from the following companies or individuals.

Cofactors and Enzymes. ATP and TPN were from Sigma Chemical Co., St. Louis, Mo. Hexokinase and glucose 6-phosphate dehydrogenase were from Boehringer-Mannheim Corp., New York, N. Y. Before use, the enzymes were extensively dialyzed against cold distilled water to remove $(NH_4)_2SO_4$.

Lipids. Cholesterol was from Sigma; dicetyl phosphate, K & K Labs, Plainview, N. J.; stearylamine, Fisher Scientific Co., St. Louis, Mo.; egg lecithin and beef brain sphingomyelin, Pierce Chemical Co., Rockford, Ill.; and beef phosphatidylserine, Applied Science Laboratories, State College, Pa. Synthetic lecithins and *Azotobacter agilis* phosphatidylethanolamine were kindly provided by Professor L. L. M. van Deenen, State University, Utrecht, The Netherlands, and Professor J. Law, Department of Biochemistry, University of Chicago, respectively. All phospholipids were routinely checked for purity by chromatography on silica gel thin-layer plates with chloroform–methanol–water (65:35:4, v/v) as solvent system and visualization by iodine vapor or ninhydrin spray.

Immunologic Reagents and Assays. Sheep and beef blood were obtained from the Colorado Serum Co., Denver, Colo., or from a local abattoir. Sheep hemolysin (rabbit antisheep erythrocyte serum) was purchased from Difco Laboratories, Detroit, Mich., and decomplemented by heating at 56° for 30 min. The source of complement was fresh guinea pig serum which had been absorbed against sheep erythrocytes to remove endogenous antisheep erythrocyte antibodies (Mayer, 1961). It was subsequently dialyzed against cold VBS[2] to reduce the amount of endogenous glucose (Haxby *et al.*, 1968). After dialysis, the guinea pig serum was titrated for complement activity ($C'H_{50}$ units) with sensitized sheep erythrocytes by the method of Mayer (1961).

Preparation, Extraction, and Fractionation of Membranes. In essence, membranes from sheep and beef erythrocytes were isolated by the method of Dodge *et al.* (1963) and extracted by the procedure of Bligh and Dyer (1959). Details of the isolation and fractionation scheme, used in this laboratory, have been described previously (Haxby *et al.*, 1968). During the course of the present investigation, three minor modifications were introduced which reduced the time needed for isolation of the appropriate fractions and also permitted work-up of larger quantities of blood. (1) After equilibration with the 310 imOsm phosphate buffer, the packed cells were lysed by the addition of 25 (instead of 15) volumes of 20 imOsm phosphate buffer. (2) After washing of the membrane preparation with the hypotonic buffer, overnight dialysis against cold distilled water was used to remove nonlipid materials which contain phosphate. (3) The step in the fractionation procedure involving hexane addition was omitted. Instead, after separation of the extract into an upper (methanol–water) and lower (chloroform) phase by centrifugation, the former (along with some interfacial material and a small amount of lower phase) was removed by aspiration. The lower phase was then taken to dryness under reduced pressure at *ca.* 40°. The residue was redissolved in chloroform to yield fraction IIa (Haxby *et al.*, 1968) and its phospholipid concentration was determined by analysis for total phosphate using the method of Gerlach and Deuticke (1963).

Liposome Preparation. Liposomes were made from fraction IIa, or an artificial mixture of pure lipids, by the "micro method" employed in earlier studies (Kinsky *et al.*, 1968; Demel *et al.*, 1968; Haxby *et al.*, 1968; Alving *et al.*, 1969). An aliquot (in chloroform), containing 2 μmoles of phospholipid, was added to 10-ml conical flasks. In the case of the artificial lipid mixtures, sufficient quantities of chloroform solutions of cholesterol, and dicetyl phosphate or stearylamine, were also added to give the molar ratios indicated in the figure and table legends. The solvent was taken off with a rotary evaporator at *ca.* 40°, under reduced pressure, and the flasks placed in a desiccator, under continuous evacuation (0.05 mm), for 1 hr to ensure that the lipids were dried completely. The lipids were then dispersed with the aid of glass beads (0.2 mm in diameter) and a Vortex mixer in 0.2 ml of 0.3 M glucose . The liposome preparation was subsequently dialyzed for 1.5 hr against 150 ml of 0.075 M KCl–0.075 M NaCl to remove most of the untrapped glucose-

[1] Although we have used the terms, Forssman hapten and antigen, interchangeably in the text, the following "classical" distinctions should be noted. Forssman antigen is the material present in boiled sheep erythrocyte membranes (among other sources) which elicits the appearance in rabbit serum of antibodies that are generally considered responsible for sensitizing sheep erythrocytes to the lytic action of complement. Forssman hapten is the material isolated from sheep erythrocyte membranes (among other sources) which inhibits the ability of the rabbit antiserum and complement to cause hemolysis of sheep erythrocytes. It is very probable that Forssman antigen and the hapten used in this study are identical, but this has not yet been demonstrated conclusively.

[2] In addition to standard abbreviations, the following have been used: VBS, Veronal-buffered saline prepared according to Mayer (1961); PC, PE, and PS, phosphatidylcholine, -ethanolamine, and -serine, respectively; SM, sphingomyelin; CHOL, cholesterol, DCP, dicetyl phosphate; SA, stearylamine. Also, as in the preceding paper (Alving *et al.*, 1969), we occasionally refer to liposomes prepared from fraction IIa as "IIa liposomes." Those prepared from a lipid mixture containing SM (or PC), CHOL, and DCP (or SA), *plus* either fraction IIb or the pure Forssman hapten, are called "IIb liposomes" or "Forssman liposomes." Liposomes made from the lipid mixtures only (*i.e.*, without incorporation of any antigen) are designated "sphingomyelin–cholesterol" or "lecithin–cholesterol" liposomes.

TABLE I: Release of Trapped Glucose from Different Liposome Preparations by Various Procedures.[a]

Procedure	Trapped Glucose (mμmoles) Released from 5 μl of Liposomes Made from					
	Beef IIa	Sheep IIa	PC–CHOL–DCP	PC–CHOL–DCP + Hapten	SM–CHOL–DCP	SM–CHOL–DCP + Hapten
1% Triton	139	147	171	137	175	131
2% Triton	142	152	172	138	191	150
AS, C′	7	91	33	78	3	165
$CHCl_3$, 10% Triton	149	158	168	141	327	247

[a] Liposomes were prepared from beef or sheep fraction IIa, and from lipid mixtures containing phospholipid (SM or PC), CHOL, and DCP in molar ratios of 2:1.5:0.22, respectively. Forssman hapten was added, where indicated, to give a ratio of 5 μg of hapten/μmole of phospholipid. Amounts of rabbit antiserum (AS) and guinea pig serum (C′) used in the spectrophotometric assay were 30.1 μl (of a 1:10 dilution) and 44.9 μl (containing 9.9 $C'H_{50}$ units), respectively. See text for additional details

Evidence has been published previously which suggests that antigenic material is actually incorporated into the liposomal membrane; *i.e.*, it must be present during liposome preparation to confer immune sensitivity to liposomes made from pure lipids (Alving *et al.*, 1969). This was accomplished in the present investigation by the addition of Forssman hapten (dissolved in equal parts of chloroform and methanol) to the lipids in the flask before removal of the organic solvents (see above). The exact quantity of antigen was adjusted to meet the requirements of the particular experiment and is specified in the appropriate legend.

Antibody Binding. This was determined by the method described in the preceding paper (Alving *et al.*, 1969); details are given in the legends to Figures 9 and 10.

Liposome Assay. Loss of glucose from liposomes was again followed spectrophotometrically by the change in 340-mμ absorbance indicating reduction of TPN in the presence of hexokinase, glucose 6-phosphate dehydrogenase, and the necessary cofactors. The stock assay reagent contained: 100 mM Tris-HCl (pH 7.5), 64 mM NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 2 mM ATP, 1 mM TPN, and approximately 80 μg of hexokinase and 40 μg of glucose 6-phosphate dehydrogenase per ml. This assay reagent is different from that used in earlier studies (Haxby *et al.*, 1968; Alving *et al.*, 1969) in two respects. (1) Replacement of 4.9 mM Veronal by Tris, as above, provided greater buffering capacity. (2) To compensate for the increase in ionic strength introduced by the change in buffers, the concentration of NaCl (originally, 145 mM) was reduced.

Cuvets (10 mm light path) initially contained 0.5 ml of the assay reagent, the indicated quantities of guinea pig serum and rabbit antiserum (diluted 1:10 with VBS), and sufficient VBS to give a final volume of 1.0 ml. The absorbance was determined at "zero time" and 30 min after addition of the liposomes (normally 5 μl of dialyzed liposome preparation were added to start the reaction). The difference between the final and initial values was a measure of the amount of marker released in the presence of antiserum and complement *plus* the contributions to the absorbancy from light scatter by the liposomes and any untrapped glucose contaminating the preparation. To correct for the latter variables, cuvets were set up which were identical with the above, but without the rabbit and guinea pig serum, and the difference in absorbance, before and approximately 5 min after liposome addition, was determined.

In the following section, the results are expressed as the per cent of trapped glucose released from the liposomes in the presence of antiserum and complement after 30-min incubation at room temperature (*ca.* 22°). When liposomes were made from sheep or beef fraction IIa, or from lecithins and cholesterol, the available trapped marker (equivalent to the total *minus* untrapped glucose in the liposome preparation) was determined essentially by the procedure described in previous papers (Kinsky *et al.*, 1968; Demel *et al.*, 1968; Haxby *et al.*, 1968; Alving *et al.*, 1969). Two cuvets were prepared: the "experimental" contained 0.5 ml of VBS and 0.5 ml of assay reagent, whereas the "control" contained 0.5 ml of VBS and 0.5 ml of assay reagent from which TPN had been omitted. The absorbancy of each was measured 5 min after liposome addition and the difference [(+TPN) − (−TPN)] was used to calculate the amount of untrapped marker. The total glucose was determined from the difference between the absorbancies of the experimental and control cuvets 5 min after addition of Triton X-100 (0.1 ml of a 10% solution prepared in 0.1 M Tris, pH 8).

Under the above conditions, the final concentration of Triton was *ca.* 1%. Table I shows that this was sufficient to release all of the trapped marker from liposomes made with IIa fractions, or egg lecithin and cholesterol (see also Kinsky *et al.*, 1968). Thus, insignificant amounts of glucose were liberated from these liposomes upon addition of another 0.1-ml aliquot of Triton to give a final concentration of 2%. However, a twofold change in detergent concentration did produce an appreciable increase in the amount of marker released from liposomes prepared with sphingomyelin and cholesterol. Moreover, saturating amounts of antiserum and complement were more effective than Triton in releasing glucose from sphingomyelin–cholesterol liposomes which had been sensitized with Forssman antigen. These results necessitated development of an alternative method to determine the total amount of glucose (trapped *plus* untrapped) present in these liposome preparations. In the procedure which was finally employed, an appropriate amount of the sphingomyelin–cholesterol liposome preparation (*ca.* 3 μl) was added

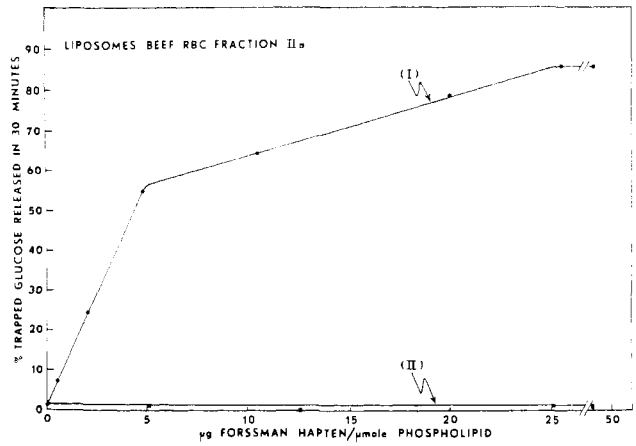

FIGURE 1: Effect of Forssman hapten on the immune sensitivity of beef IIa liposomes. In the experiment shown in curve I, liposomes were prepared from mixtures of beef fraction IIa and appropriate quantities of Forssman hapten to give the ratios indicated on the abscissa. Glucose release was determined by the spectrophotometric assay in the presence of 30.1 μl of a 1:10 dilution of rabbit antiserum and 115 μl of guinea pig serum (containing 16.8 $C'H_{50}$ units of complement). In the experiment shown in curve II, glucose release was determined under identical conditions from liposomes prepared with beef fraction IIa alone; aliquots of a dispersion of Forssman hapten (containing 5 μg/ml of VBS) were added to the assay cuvets to give the ratios shown on the abscissa. See text for additional details.

to duplicate tubes and thoroughly mixed with 0.5 ml of chloroform. The solvent was removed on a rotary evaporator in 0.1 ml of the 10% Triton solution using a Vortex mixer. Water (0.4 ml) was added followed by 0.5 ml of complete assay reagent (+TPN) to one of the tubes and 0.5 ml of incomplete assay reagent (−TPN) to the other. The difference in absorbancies was determined after incubation for 5 min at room temperature. This value, when corrected for the absorbancy difference due to untrapped glucose (measured by the method described in the preceding paragraph) was used to calculate the amount of trapped marker.

The efficacy of this procedure is indicated in Table I. In this experiment, the amount of trapped glucose in sphingomyelin–cholesterol liposomes, measured by the chloroform–Triton method, was approximately 1.7 times the value obtained by direct "lysis" of the liposomes with Triton. Accordingly, the apparent glucose released from sphingomyelin–cholesterol liposomes, sensitized with Forssman antigen, by antiserum and complement decreased from "126" % (*i.e.*, 165/131 × 100) to 67% (*i.e.*, 165/247 × 100) of the trapped marker. However, the amounts of glucose released by the two methods were identical in the case of liposomes prepared with egg lecithin, beef, or sheep fraction IIa. This further supports the conclusion that Triton treatment *per se* suffices to liberate all of the trapped marker from these liposomes (see above).[3]

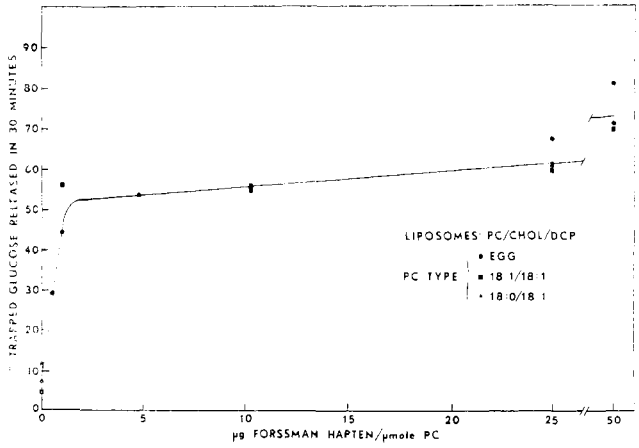

FIGURE 2: Effect of Forssman hapten on the immune sensitivity of lecithin–cholesterol liposomes. Liposomes were prepared from mixtures containing PC, CHOL, and DCP in molar ratios of 2:1.5:0.22, respectively. The abscissa indicates the amount of Forssman hapten per micromole of PC added to the mixture. The synthetic lecithins were 1,2-dioleoyl- and 1-stearoyl-2-oleoylphosphatidylcholine (abbreviated 18:1/18:1 and 18:0/18:1 PC, respectively). Glucose release was determined by the spectrophotometric assay in the presence of 30.1 μl of a 1:10 dilution of rabbit antiserum and 50.9 μl of guinea pig serum (containing 8.8 $C'H_{50}$ units of complement).

Results

Sensitization by Forssman Hapten. Beef erythrocytes, unlike sheep erythrocytes, do not contain Forssman antigen. Table I corroborates earlier experiments (Haxby *et al.*, 1968) which have demonstrated that liposomes prepared from beef erythrocyte fraction IIa, unlike those made with the corresponding fraction from sheep erythrocytes, release very little trapped glucose when incubated with rabbit antisheep erythrocyte serum and complement. If the Forssman system is involved in the response of the sheep IIa liposomes, as suggested by the above observations, then pure Forssman hapten might be expected to confer immune sensitivity to beef IIa liposomes. The experiment illustrated in curve I of Figure 1 confirms this prediction. It should be noted (Figure 1, curve II) that Forssman hapten does not sensitize liposomes if it is simply added to the assay system, *i.e.*, it must be added to the lipid mixture from which the liposomes are generated. In this regard, Forssman hapten resembles the antigenic material in fraction IIb isolated from sheep erythrocytes. This crude fraction, which contains methanol–water-soluble components, is also able to sensitize liposomes. However, in contrast to either beef or sheep fraction IIa, it cannot form liposomes alone because it lacks the major lipids (chloroform soluble) of the cell membrane (Haxby *et al.*, 1968; Alving *et al.*, 1969).

[3] Additional experiments (not reported) have shown that the chloroform–Triton procedure releases as much glucose from sphingomyelin–cholesterol liposomes as does disruption of the liposomes by prolonged sonication. Furthermore, the unexpected stability of these liposomes toward Triton is also apparent when stearylamine is used, instead of dicetyl phosphate, to generate positively charged liposomes. However, the liposomes become more sensitive to Triton when a portion of the sphingomyelin is replaced by phosphatidylethanolamine or phosphatidylcholine; *i.e.*, the ratio of the amount of glucose released by Triton alone to that liberated by chloroform–Triton approaches 1. The fact that this occurs with liposomes containing sphingomyelin, phosphatidylethanolamine, phosphatidylserine, and cholesterol in molar ratios (1.3:0.7:0.22:1.5, respectively) which approximate the composition of sheep and beef erythrocyte membranes (Rouser *et al.*, 1968), is consistent with the observation that liposomes prepared from the corresponding IIa fraction release all of their trapped glucose in the presence of 1–2% Triton (Table I).

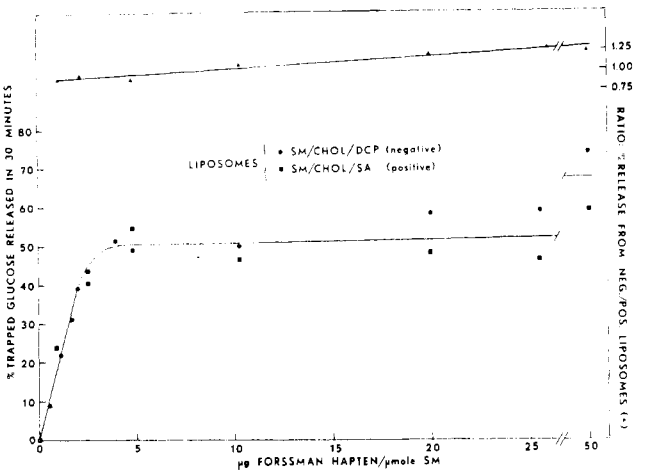

FIGURE 3: Effect of Forssman hapten on the immune sensitivity of sphingomyelin–cholesterol liposomes. Liposomes were prepared from mixtures containing SM, CHOL, and DCP (or SA) in molar ratios of 2:1.5:0.22, respectively. The abscissa indicates the amount of Forssman hapten per micromole of SM added to the mixture. Glucose release was determined by the spectrophotometric assay in the presence of 30.1 μl of a 1:10 dilution of rabbit antiserum and 44.9 μl of guinea pig serum (containing 9.4 $C'H_{50}$ units of complement). The right ordinate gives the ratio of the per cent glucose released from negative liposomes (prepared with DCP) to the per cent marker liberated from positive liposomes (prepared with SA).

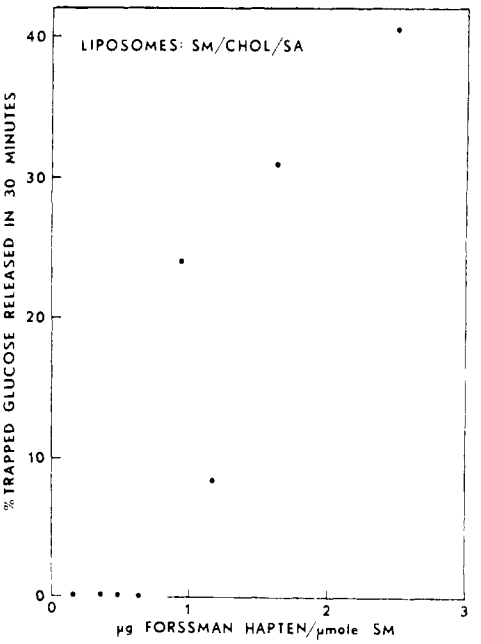

FIGURE 4: Effect of Forssman hapten on the immune sensitivity of sphingomyelin–cholesterol liposomes. Procedure identical with that recorded in the legend to Figure 3 except that glucose release was determined by the spectrophotometric assay in the presence of 11.1 μl of a 1:10 dilution of rabbit antiserum and 44.9 μl of guinea pig serum (containing 9.4 $C'H_{50}$ units of complement).

Forssman hapten also sensitizes liposomes, prepared from artificial lipid mixtures, to rabbit antiserum and complement. Figure 2 compares the response of liposomes, made with natural (egg) lecithin, with the behavior of liposomes generated from two synthetic phosphatidylcholines. No appreciable difference between the various liposome preparations is apparent. The effect of Forssman hapten on sphingomyelin–cholesterol liposomes is described in Figure 3. In this experiment, dicetyl phosphate and stearylamine were used as the charged amphiphile to make negatively and positively charged liposomes, respectively (Bangham *et al.*, 1965; Bangham, 1968). Rabbit antiserum, in the presence of complement, released essentially the same percentage of glucose from both classes of liposomes. The possible significance of these observations, as they pertain to complement mechanism, is discussed later.

Near maximum sensitization of lecithin–cholesterol liposomes (Figure 2) or sphingomyelin–cholesterol liposomes (Figure 3) occurs with approximately 5 μg of the pure Forssman hapten per μmole of phospholipid. Assuming a molecular weight of 1300 for the compound, this corresponds to a ratio of 1 molecule of antigen for every 250 molecules of phospholipid (or 450 molecules of phospholipid and cholesterol) in these liposome preparations. It is of some interest to compare the potency of the hapten with sheep fraction IIb. In the case of fraction IIb, near maximum sensitization of liposomes, prepared with either lecithin–cholesterol or sphingomyelin–cholesterol mixtures, required between 150 and 250 μg per μmole of phospholipid (Haxby *et al.*, 1968; Alving *et al.*, 1969). These quantitative considerations are consistent with the previous suggestions that the active component in fraction IIb may be Forssman antigen. Additional observations, which also support this conclusion, are described below.

As noted earlier (see Discussion of Table I), sphingomyelin–cholesterol liposomes are far more resistant to the disruptive effects of Triton than are lecithin–cholesterol liposomes. During the course of this study, it was observed that these liposomes also show less "endogenous" glucose release. Thus, as indicated in Table I, unsensitized sphingomyelin–cholesterol liposomes lose only 1% (usually less) of the available trapped marker in the presence of antiserum and complement, whereas unsensitized lecithin–cholesterol liposomes may release as much as 20% under the same conditions. This property of sphingomyelin–cholesterol liposomes made feasible experiments designed to test the effect of very small amounts of Forssman hapten on the response of liposomes. The results illustrated in Figure 4 suggest that sensitization by the antigen may be a threshold phenomenon which requires attainment of a "critical" antigen to phospholipid ratio before it becomes apparent.

The preceding experiments show that it is possible to manufacture a model membrane, in which the essential features of immune lysis apparently can be mimicked, from lipids of known structure and composition. It has also been possible to prepare immunologically responsive liposomes by incorporating the Forssman hapten into lipid mixtures which are more complex than those just described. However, one of the objects of this investigation has been to devise the "simplest" liposome which would release glucose in the presence of antiserum and complement. Unfortunately, further efforts along these lines have encountered several experimental obstacles. For example, it has not yet been possible to omit cholesterol. Thus, mixtures, containing various proportions of sphingomyelin (or phosphatidylethanolamine) and dicetyl phosphate (or stearylamine), do not

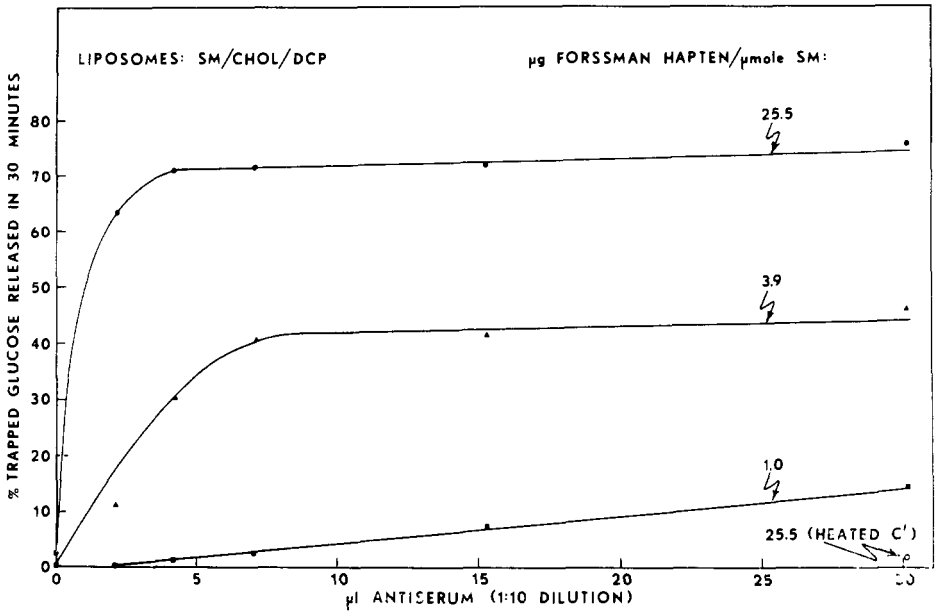

FIGURE 5: Effect of Forssman hapten on antiserum requirement for glucose release from sphingomyelin–cholesterol liposomes. Liposomes were prepared from mixtures containing SM, CHOL, and DCP in molar ratios of 2:1.5:0.22, respectively, and the amounts of Forssman hapten specified in the figure. Glucose release was determined by the spectrophotometric assay in the presence of 50.9 μl of guinea pig serum (containing 7.4 $C'H_{50}$ units) and varying amounts of a 1:10 dilution of rabbit antiserum as indicated on the abscissa. The liposomes, containing 25.5 μg of Forssman hapten per micromole of SM, were also assayed in the presence of 50.9 μl of guinea pig serum which had been heated at 56° for 30 min.

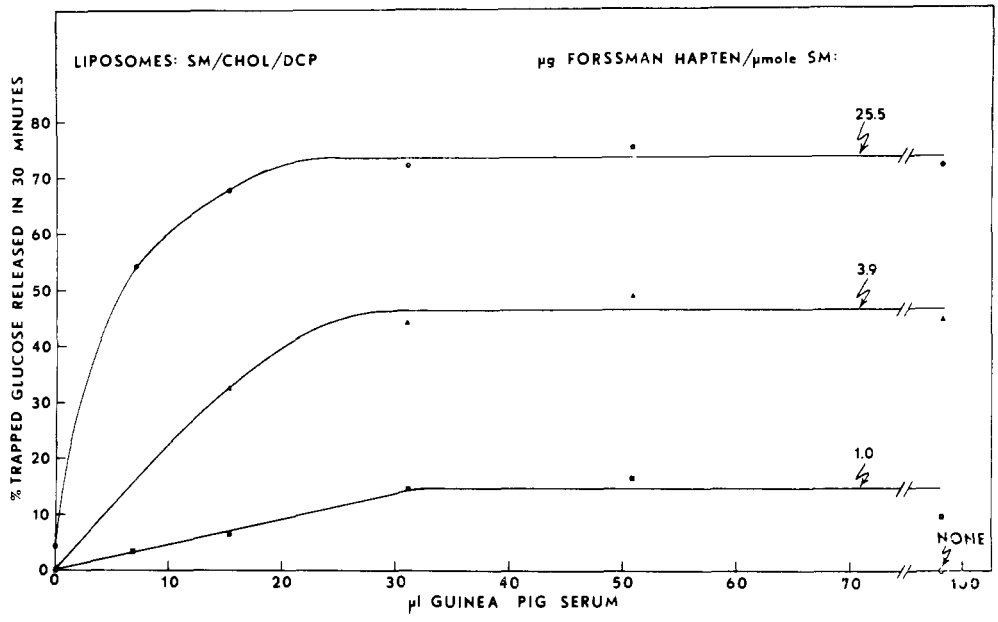

FIGURE 6: Effect of Forssman hapten on complement requirement for glucose release from sphingomyelin–cholesterol liposomes. Procedure identical with that recorded in the legend to Figure 5 except that glucose release was determined by the spectrophotometric assay in the presence of 30.1 μl of a 1:10 dilution of rabbit antiserum and varying amounts of guinea pig serum as indicated on the abscissa. The guinea pig serum used in this experiment had an activity of 146 $C'H_{50}$ units/ml.

readily form liposomes with appreciable quantities of trapped marker. Although this does not apply to liposomes made from mixtures of egg lecithin and dicetyl phosphate, these liposomes are not sufficiently stable under the conditions of assay and lose nearly all their marker when, for example, rabbit antiserum is absent or if decomplemented (*i.e.*, heated) guinea pig serum is used. For similar reasons, phosphatidylserine–cholesterol liposomes, which do not require addition of any other charged amphiphile because this function is also served by the phospholipid (see Bangham, 1968), could not

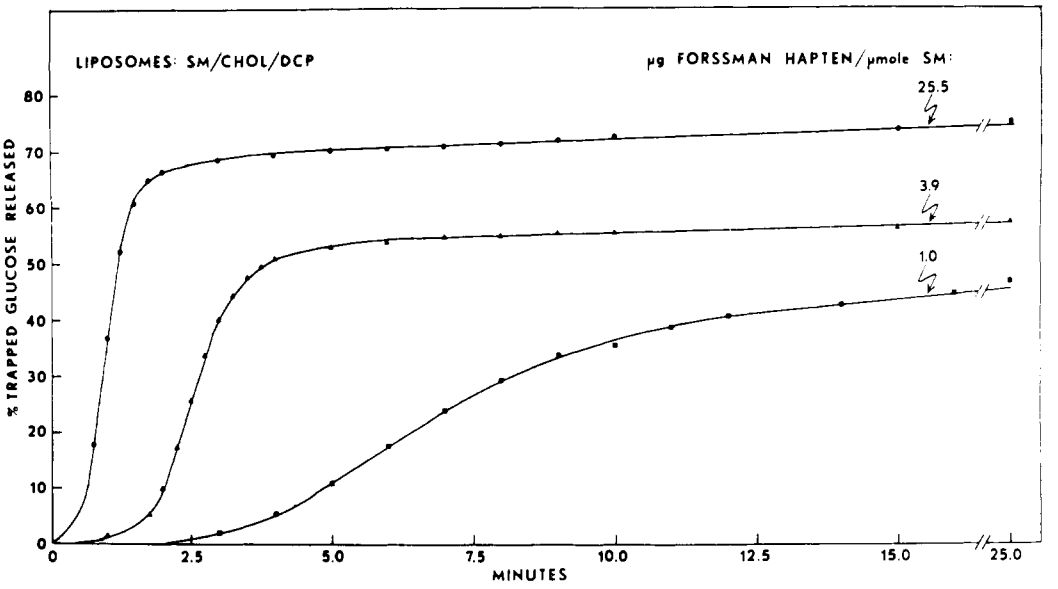

FIGURE 7: Effect of Forssman hapten on the rate and extent of glucose release from sphingomyelin–cholesterol liposomes. Liposomes were prepared from mixtures containing SM, CHOL, and DCP in molar ratios of 2:1.5:0.22, respectively, and the amounts of Forssman hapten specified in the figure. Glucose release was determined by the spectrophotometric assay at various times after liposome addition to cuvets containing 30.1 μl of a 1:10 dilution of rabbit antiserum and 50.9 μl of guinea pig serum (7.4 $C'H_{50}$ units).

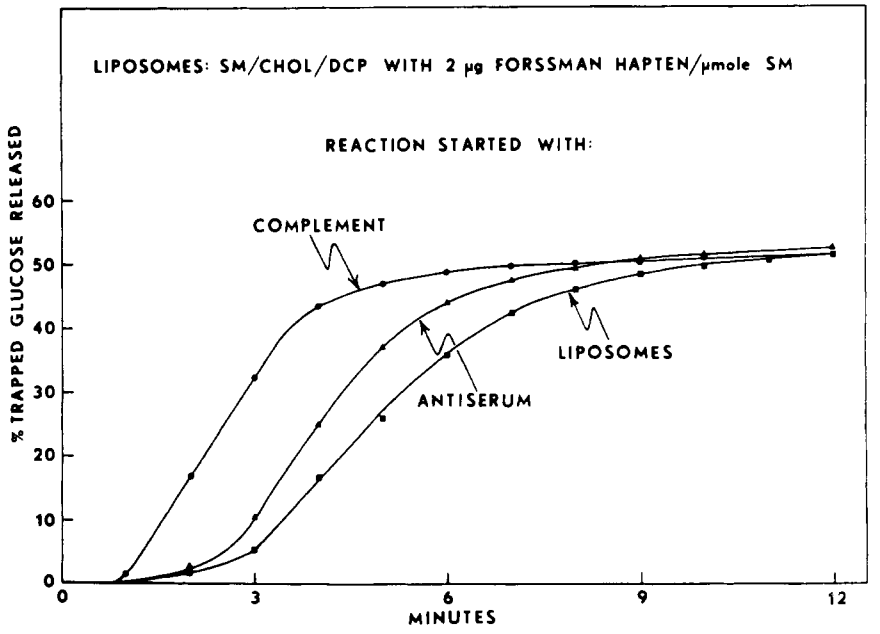

FIGURE 8: Effect of order of addition on the rate of glucose release from sphingomyelin–cholesterol liposomes sensitized with Forssman hapten. Liposomes were prepared from mixtures containing SM, CHOL, and DCP in molar ratios of 2:1.5:0.22, respectively, and the amount of Forssman hapten specified in the figure. Glucose release was determined by the spectrophotometric assay in the presence of 30.1 μl of a 1:10 dilution of rabbit antiserum and 50.9 μl of guinea pig serum (containing 7.4 $C'H_{50}$ units of complement). The assay was conducted by the "standard" procedure, used for all of the other experiments, in which the liposome preparation (5 μl) was added last. Modifications of this assay were used, as indicated in the figure, in which the reaction was started either with the guinea pig serum (complement) or the rabbit antiserum. Cuvets were incubated 5 min at room temperature (*ca.* 21°) before addition of the terminal reactant.

be employed. In the latter case, it proved difficult to demonstrate an effect of Forssman hapten incorporation on the response of the liposomes because of the high endogenous level of glucose release, manifested by unsensitized liposomes, either in the presence or absence of rabbit and guinea pig serum. Up to now, the sphingomyelin (or lecithin)–cholesterol liposomes described above are the simplest that we have yet devised as objects for the study of complement mechanism.

Effect of Antiserum and Complement Concentration and the Kinetics of Glucose Release. The effect of rabbit antiserum

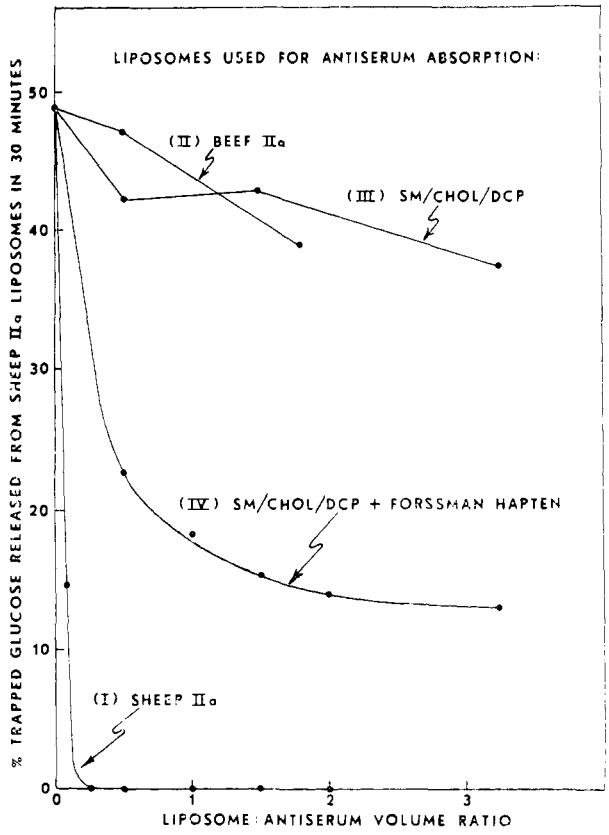

FIGURE 9: Effect of preincubation with different liposome preparations on the ability of rabbit antiserum to cause glucose release from sheep fraction IIa liposomes. Liposomes were prepared from beef or sheep fraction IIa, and a lipid mixture containing SM, CHOL, and DCP in molar ratios of 2:1.5:0.22, respectively. Forssman hapten was added, where indicated, to give a ratio of 5 μg of hapten/μmole of SM. The lipids were dispersed with an isotonic saline solution (0.075 M NaCl–0.075 M KCl) instead of glucose. Absorption was performed in the presence of varying quantities (0–98 μl) of liposomes, 30.1 μl of a 1:10 dilution of rabbit antisheep erythrocyte serum, and sufficient VBS to give a final volume of 0.2 ml. The abscissa indicates the ratio of liposome to diluted antiserum volume. After 15 min at room temperature 0.6 ml of VBS and 0.8 ml of complete assay reagent were added, and the liposomes were removed by centrifugation at 28,000g for 15 min. Guinea pig serum (115 μl, containing 16.8 $C'H_{50}$ units of complement) and sheep fraction IIa liposomes (5 μl), which had been swollen in glucose, were added to 0.8 ml of the supernatant solution. Absorbancy at 340 mμ was determined before, and 30 min after, addition of the complement and liposomes. The ordinate indicates the per cent marker released after correction for volume changes, untrapped glucose in the guinea pig serum and liposome preparation, and any loss occurring in the absence of antiserum.

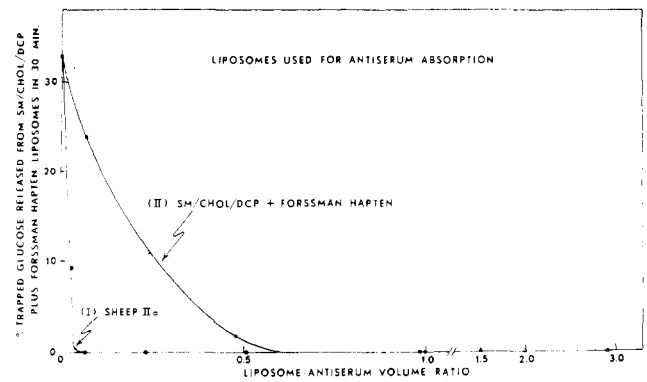

FIGURE 10: Effect of preincubation with different liposome preparations on the ability of rabbit antiserum to cause glucose release from sphingomyelin–cholesterol liposomes sensitized with Forssman hapten. Procedure identical to that recorded in the legend to Figure 9 with the following exceptions. (a) A total of 15.3 μl of a 1:10 dilution of rabbit antiserum was absorbed with the liposome preparations indicated in the figure, and (b) glucose release was determined from liposomes made with SM, CHOL, and DCP (molar ratios of 2:1.5:0.22, respectively) which had been sensitized with 5 μg of Forssman hapten/μmole of SM.

concentration on the loss of marker from sphingomyelin–cholesterol liposomes, prepared with different amounts of Forssman hapten, is shown in Figure 5. In the presence of excess guinea pig serum, less antiserum is required to produce a given percentage of glucose release when more of the antigen is incorporated. It should be noted that, under the conditions of assay, antiserum *per se* does not cause loss of marker if the guinea pig serum was heated to inactivate complement. The converse experiment, describing the effect of guinea pig serum concentration on sensitized sphingomyelin–cholesterol liposomes, is illustrated in Figure 6. In the presence of excess antiserum, less complement is needed for a given percentage of glucose release when greater amounts of Forssman hapten are included. These results suggest that the extent of marker loss depends upon the number of antigen–antibody complexes which are formed within the liposomal membrane. The latter in turn initiates the complement sequence which ultimately leads to membrane damage. Formation of antibody–antigen complexes would be favored either by incorporation of more antigen or higher concentrations of antiserum, whereas activation of C1 by the complexes (the first step in the complement sequence; see, for example, Müller-Eberhard, 1968) would be favored by higher concentrations of guinea pig serum.[4]

The above interpretation is also consistent with previous experiments indicating that antibody must be bound by the liposomes before complement can be fixed (Alving *et al.*, 1969), and by the time course of glucose release illustrated in Figures 7 and 8. As in the case of sheep IIa liposomes (Haxby *et al.*, 1968), a lag phase precedes the release of marker. The lag phase is shortest with liposomes that contain the highest amount of antigen; these liposomes also lose their marker at the fastest rate (Figure 7). Furthermore, the lag phase is shortest when glucose release is initiated by the addition of complement, and longest when the liposomes were added last (Figure 8). However, the subsequent rate and extent of glucose release is essentially the same when started with either antiserum, complement or liposomes.

Antibody Binding by Liposomes. Previous studies (Alving

[4] The present findings may also explain some anomalous properties of liposomes prepared from different lots of sheep fraction IIa. Although these liposomes generally release between 50 and 70% of their trapped glucose in the presence of saturating levels of rabbit antiserum and guinea pig serum, we have occasionally encountered preparations which give as little as 30, or as much as 90% release. This may be a consequence of different antigen concentrations in sheep IIa fractions which were isolated at various times and from different lots of erythrocytes. For this reason, we have found it necessary to perform titration curves with each preparation of sheep fraction IIa to determine the sensitivity of liposomes made from it to antiserum and complement.

et al., 1969) have demonstrated that sheep IIa liposomes can bind all the antibodies which are responsible for the release of glucose from IIb liposomes. However, absorption of rabbit antiserum with IIb liposomes did not remove all of the antibodies required for marker release from IIa liposomes. The following experiments indicate that the Forssman hapten behaves similarly to sheep fraction IIb in this regard.

Figure 9 (curve I) shows that, as expected, sheep fraction IIa liposomes can bind all the antibodies in the rabbit antiserum which are necessary to induce glucose release from the same liposomes. In contrast, beef fraction IIa liposomes do not bind significant amounts of the antibodies under conditions which are conducive to complete absorption by sheep fraction IIa liposomes (compare curve II with curve I at a liposome to antiserum volume ratio of 0.25). The latter is consistent with earlier observations that rabbit antisheep erythrocyte serum does not promote loss of marker from the beef IIa liposomes in the presence of complement. Also, sphingomyelin–cholesterol liposomes have essentially no effect over the concentration range employed in this experiment (curve III). However, when the sphingomyelin–cholesterol liposomes are sensitized with Forssman hapten, they are able to bind an appreciable portion of the antibodies required for glucose release from sheep fraction IIa liposomes (curve IV). The important point to note is that binding by sensitized sphingomyelin–cholesterol liposomes is not complete. Thus, even after absorption with these liposomes at a liposome to antiserum volume ratio of 3.25, the rabbit antiserum retains an appreciable portion of its capacity to release marker from sheep IIa liposomes.

The converse experiment, in which glucose release from sensitized sphingomyelin–cholesterol liposomes was measured, is illustrated in Figure 10. Curve II shows that Forssman liposomes effectively bind all the antibodies in the rabbit antiserum that are required for loss of marker from these liposomes. Low concentrations of sheep IIa liposomes also absorb the same antibodies (curve I). The latter is not surprising in view of the fact that the Forssman hapten was originally isolated from sheep erythrocyte cell membranes.

Discussion

The experiments reported in this paper show that addition of pure Forssman hapten to a lipid mixture of known composition confers immune sensitivity to the resultant liposome preparation. These experiments further indicate that the response of liposomes, prepared from the chloroform-soluble fraction (IIa) of sheep erythrocyte membranes, may be in part initiated by the interaction of antibody with Forssman antigen. In this connection, it should be noted that our original efforts to prepare a model membrane, which would duplicate the essential features of immune lysis, were carried out with the lipid fraction of sheep erythrocytes on the assumption that the amphipathic properties of Forssman antigen might favor its proper insertion into the bilayer lamellae of the liposomes (Haxby *et al.*, 1968; Alving *et al.*, 1969).

However, it is apparent from the antibody binding experiments that the response of the sheep IIa liposomes need not involve only Forssman antigen with the structure of the material used in this study. The extensive investigations carried out in the laboratories of Yamakawa and Rapport have demonstrated the existence of numerous other antigens which are structurally related to this Forssman hapten. For example, globoside I or cytolipin K, cytolipin R, cytolipin H, glucocerebroside, and galactocerebroside, also contain polar antigenic determinants (*i.e.*, the carbohydrates) which are covalently linked to a nonpolar (*i.e.*, ceramide) residue. The quantitative assay of these antigens by immunological procedures (either as haptenic inhibitors or by complement fixation) almost invariably requires prior admixture with "auxiliary lipids" (see, for example, Makita *et al.*, 1966; Rapport *et al.*, 1967). This point is mentioned because the procedure, by which antigen is combined with these lipids (*e.g.*, lecithin–cholesterol, sphingomyelin–cholesterol), resembles to some extent the method for preparing liposomes: solution in organic solvents, evaporation to dryness, and dispersion in an aqueous environment. This parallelism raises the possibility, currently under investigation, that the other ceramide antigens might also yield liposomes which would release trapped glucose in the presence of the appropriate antibodies and complement. Indeed, this may be a property of amphipathic antigens in general because preliminary experiments have shown that immunologically sensitive liposomes can be prepared by incorporation of some pure lipopolysaccharides isolated from *Salmonella minnesota* rough mutants (S. C. Kinsky and O. Lüderitz, unpublished observations).

Perhaps the most significant product of this investigation is the suggestion that lipids arranged in bilayer configuration alone may serve as substrate for complement. The evidence in favor of this general structure for liposomes has recently been reviewed in detail (Bangham, 1968). These experiments also imply that cell membranes may not contain any unique and specific endogenous receptor sites (protein or carbohydrate) for components of the complement sequence. This does not preclude the possibility that receptors for some of the complement proteins are present in the rabbit and guinea pig serum, and that these have interacted with the liposomes during assay. The capacity to exercise control over the composition of the liposomes (by altering the composition of the lipid mixture from which they were generated) has, during the course of this study, led to some observations which may bear on the question of complement mechanism. The fact that liposomes, prepared with either a glycerol- or sphingosine–phospholipid, are both rendered immunologically sensitive by incorporation of Forssman hapten again argues against the possibility that lysolecithin or lysophosphatidylethanolamine (originating from membrane lipids) may be involved in the lytic step. Thus far, we have not obtained any convincing evidence to indicate that glucose release occurs as a consequence of degradation of the lipids which constitute the model membrane. Although liposomes, prepared without cholesterol, could not be checked for their immune sensitivity because they are unstable under our assay conditions, it seems very unlikely that enzymatic attack on this molecule plays a role. Gram-negative bacteria, which lack sterols, are susceptible to lysis in the presence of the appropriate antibodies and complement. In fact, the present experiments suggest that the net charge on the liposomal membrane may have only minor significance, if any. The available data are at least consistent with the working hypothesis that the sequential action of the complement "cascade" (for review, see Müller-Eberhard, 1968) leads to the exposure of a hydrophobic group(s) on the terminal component(s) (*i.e.*, C8 and/or

C9), and that the latter disrupts the membrane by rupture of noncovalent bonds between the membrane lipids.

Acknowledgments

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References

Alving, C. R., Kinsky, S. C., Haxby, J. A., and Kinsky, C. B. (1969), *Biochemistry 8*, 1582.

Bangham, A. D. (1968), *in* Progress in Biophysics and Molecular Biology, Butler, J. A. V., and Noble, D., Ed., New York, N. Y., Pergamon, p 29.

Bangham, A. D., Standish, M. M., and Watkins, J. C. (1965), *J. Mol. Biol. 13*, 238.

Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37*, 911

Demel, R. A., Kinsky, S. C., Kinsky, C. B., and van Deenen, L. L. M. (1968), *Biochim. Biophys. Acta 150*, 655.

Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100*, 119.

Gerlach, E., and Deuticke, B. (1963), *Biochem. Z. 337*, 477.

Haxby, J. A., Kinsky, C. B., and Kinsky, S. C. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 300.

Kinsky, S. C., Haxby, J. A., Kinsky, C. B., Demel, R. A., and van Deenen, L. L. M. (1968), *Biochim. Biophys. Acta 152*, 174

Makita, A., Suzuki, C., and Yosizawa, Z. (1966), *J. Biochem. (Tokyo) 60*, 502.

Mayer, M. M. (1961), *in* Experimental Immunochemistry, Kabat, E. A., and Mayer, M. M., Ed., Springfield, Ill., C. C Thomas, p 133.

Müller-Eberhard, H. J. (1968), *in* Advances in Immunology, Dixon, F. J., and Kunkel, H. G., Ed., New York, N. Y., Academic, p 1.

Rapport, M. M., Cavanna, R., and Graf, L. (1967), *J. Neurochem. 14*, 9.

Rouser, G., Nelson, G. J., Fleischer, S., and Simon, G. (1968), *in* Biological Membranes, Chapman, D., Ed., New York, N. Y., Academic, p 5.