Biochimica et Biophysica Acta, 298 (1973) 158–179

BBA 76222

RELEASE OF MACROMOLECULAR MARKERS (ENZYMES) FROM LIPOSOMES TREATED WITH ANTIBODY AND COMPLEMENT

AN ATTEMPT AT CORRELATION WITH ELECTRON MICROSCOPIC OBSERVATIONS

TATESHI KATAOKA*, JOSEPH R. WILLIAMSON and STEPHEN C. KINSKY**

Departments of Pharmacology, Microbiology and Pathology; Washington University School of Medicine, St. Louis, Mo. 63110 (U.S.A.)

(Received September 4th, 1972)

SUMMARY

Antibody–complement dependent damage to liposomal model membranes has been previously investigated by measuring the release of low molecular weight markers such as glucose. To determine whether larger solutes are also released under these conditions, experiments have been performed using immunologically sensitive liposomes that contained not only trapped glucose, but also enzymes (hexokinase, glucose-6-phosphate dehydrogenase, β-galactosidase) as macromolecular markers. The largest of these enzymes (β-galactosidase) has dimensions which closely approximate the diameter of the lesions detected by negative staining in natural membranes after immune lysis. Liposomes prepared with lecithin, and either actively sensitized with globoside or passively sensitized with alkali-treated lipopolysaccharide, released the enzymes in parallel with glucose upon incubation with the appropriate antiserum and native guinea pig serum as source of complement. Immune damage to sphingomyelin liposomes was characterized by a significantly lower loss of the enzymes in comparison to the percentage of glucose released; a comparable response was manifested by liposomes prepared from sheep erythrocyte lipids. Electron microscopic examination of negatively stained lecithin liposomes, which had released the macromolecular markers, failed to reveal the characteristic lesions; these findings are consistent with evidence obtained by other laboratories suggesting that the lesions may not correspond to functional holes. Lesions were, however, consistently observed in liposome preparations that had been treated with the polyene antibiotic, filipin; this antibiotic causes appreciable loss of both glucose and enzymes from either lecithin or sphingomyelin liposomes.

Abbreviation: ONPG, *o*-nitrophenyl-β-D-galactoside.

* Present address: Wistar Institute, Philadelphia, Pa., U.S.A.

** Requests for reprints should be sent to S.C.K. at Washington University.

INTRODUCTION

In 1964, Borsos *et al.*[1] described the appearance of discrete lesions in negatively stained sheep erythrocyte membranes obtained from cells that had been lysed by rabbit antisheep erythrocyte serum (as source of antibodies) in the presence of guinea pig serum (as source of complement). These lesions were essentially circular with a diameter of 80–100 Å in the dark area where the negative phosphotungstate stain had accumulated, surrounded by a light ring. Subsequent studies (reviewed in ref. 2) indicated that these lesions were not confined to erythrocytes but could be demonstrated also in the membranes of a variety of mammalian and bacterial cells that had undergone immune cytolysis. These observations, among other lines of evidence, led Humphrey and Dourmashkin[2] to the tentative conclusion that the lesions probably correspond to the effective holes in the membrane that are responsible for cellular lysis.

Recent experiments by Polley *et al.*[3,4], have, however, cast some doubt on this interpretation. They have shown that lesions are already apparent on sheep erythrocyte membranes after the reaction involving the fifth component of complement (C5), although such cells still manifest an intact permeability barrier because neither lysis nor increased loss of cations was observed*. Furthermore, addition of the later acting complement components (C6 through C9) necessary to produce hemolysis did not cause any increment in the number of lesions over that obtained after reaction with C5. On the basis of these observations, Polley and co-workers[3,4] have emphasized the need for caution in equating the ultrastructural lesions with functional holes.

In 1968, Haxby *et al.*[5] described the formation of liposomal model membranes which, as indicated by further studies, closely mimicked the response of natural membranes to the complement system either in the presence or absence of an appropriate antibody (see ref. 6 for review). Unfortunately, in the case of such liposomes, electron microscopic investigations have yielded results that are not entirely consistent. In their investigation of the "reactive lysis" of liposomes, Lachmann *et al.*[7] obtained evidence compatible with the thesis that the lesions correspond to actual holes. They could not detect any lesions in liposomes that had been reacted with a complement reagent containing C5, C6, and C7; lesions were, however, demonstrable after the subsequent addition of C8 and C9. In contrast, Knudsen *et al.*[8] failed to find any lesions in liposomes prepared from a lipid extract of sheep erythrocyte membranes (sheep fraction IIa) that had been treated with anti-Forssman** antibodies and guinea pig serum; instead, they described the formation of lipid "globules" and a loss of the characteristic lamellar structure of liposomes. More recently, Hesketh *et al.*[9] have also examined liposomes generated from sheep erythrocyte lipids. In confirmation of Knudsen *et al.*[8], they observed the partial "disappearance" of liposomes in the presence of anti-Forssman antibodies and either human or rabbit serum as complement source; however, the liposomes that remained were covered with typical complement lesions.

* It should be noted, for readers not completely familiar with the complement system, that the nine functional protein components (C) of the classical complement sequence react in the following order to produce hemolysis of antibody-sensitized erythrocytes: C1, C4, C2, C3, C5, C6, C7, C8, C9.

** Forssman is the predominant glycolipid (ceramide) antigen that is responsible for the sensitization of sheep erythrocytes to antibody–complement; its structure has been recently established[21].

Our preliminary electron microscopic studies of antibody–complement treated liposomes have also been equivocal. In the initial paper[5] describing the formation of these immunologically responsive model membranes, we noted the appearance of lesions ("pits") in sheep fraction IIa liposomes and in lecithin–cholesterol–dicetyl phosphate liposomes that had been sensitized with bacterial lipopolysaccharide as antigen. Further experiments (Zopf, D. A. and Kinsky, S. C., unpublished observations) did not, however, warrant the conclusion that these lesions represented the holes through which glucose (employed as marker for following changes in liposomal permeability; see below) escaped from the liposomes. In the case of sheep fraction IIa liposomes, we could not establish any direct correlation between the extent of glucose loss and the number of lesions present on the liposomes; in fact, several liposome preparations did not manifest any lesions although as much as 80% of the trapped marker had been released. In the case of the lipopolysaccharide-sensitized preparations, lesions were consistently present; however, these lesions did not seem to be associated with the model membrane but rather with material that had not been incorporated into the liposomes. This material was probably free lipopolysaccharide in view of the earlier findings (reviewed in ref. 2) that the latter alone can serve as substrate for the production of lesions by an appropriate antiserum and complement source.

Before any significance could be attached to these negative findings, we considered it essential to determine if marker compounds that approached the dimensions of the lesions were also released from the liposomes in the presence of antibody–complement. In this regard, it should be noted that immune damage to liposomes has, up to now, been followed exclusively by the release of low molecular weight markers; these have been glucose[5,7], galactose[8], and chromate ions[7]. Thus, failure to correlate the presence of lesions with the extent of marker loss could be due to the fact that complement produces "holes" in these model membranes that are of sufficient size to permit only the escape of relatively small compounds.

To circumvent the limitations of this approach, the present investigation was performed utilizing liposomes of different phospholipid composition that contained, not only glucose, but also trapped enzymes as macromolecular markers; these enzymes were hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) and β-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23). For comparative purposes, we have examined, not only the effects of antibody–complement, but also the influence of filipin on the permeability of these liposomes because previous studies[10] have shown that this polyene antibiotic produces lesions that are quite similar to those observed after immune lysis. A preliminary account of these experiments has appeared[11].

MATERIALS AND METHODS

Lipids

These were obtained from the following commercial sources: beef brain sphingomyelin and egg lecithin (Pierce Chemical Co., Rockford, Ill.); cholesterol (Sigma Chemical Co., St. Louis, Mo.); dicetyl phosphate (K and K Laboratories; Plainview, N. J.); stearylamine (Fischer Scientific Co., St. Louis, Mo.). Sheep fraction IIa was isolated by the procedures described previously[5,12].

Filipin and saponin

The antibiotic complex was obtained through the courtesy of Dr George Whitfield, Upjohn Co., Kalamazoo, Mich.; stock solutions, containing 10 mg of filipin per ml of dimethylformamide, were prepared fresh as needed. Saponin (Nutritional Biochemicals Corp., Cleveland, Ohio) was purified in the following manner to remove glucose which was present as an appreciable contaminant. 10 ml of an aqueous suspension containing 1 g of saponin was dialyzed overnight against 2 l of cold distilled water. Absolute ethanol (500 ml) was then added to the sac contents and the precipitate was washed twice with 40 ml of ethanol, once with 30 ml of diethyl ether, and finally dried *in vacuo*. Stock solutions for use in the assay of total trapped markers (see below) were prepared by dissolving 100 mg of the purified material in 1 ml of 100 mM Tris, pH 7.5.

Cofactors and substrates

ATP, NADP and *o*-nitrophenyl-β-D-galactoside were purchased from Sigma.

Enzymes

Yeast hexokinase and glucose-6-phosphate dehydrogenase, and *Escherichia coli* β-galactosidase, were obtained from Boehringer-Mannheim Corp., New York, N. Y. Before use, the enzymes were extensively dialyzed against cold distilled water to remove $(NH_4)_2SO_4$; protein concentration after dialysis was approx. 7, 2, and 7 mg per ml for hexokinase, glucose-6-phosphate dehydrogenase, and β-galactosidase, respectively. The same dialyzed enzyme solutions were employed for preparation of the swelling media in which the liposomes were generated, and for preparation of the assay reagents by which marker release was determined (see below). *Clostridium perfringens* phospholipase C was purchased from Sigma; stock solutions of the enzyme (not dialyzed) were made by dissolving 1 mg in 1 ml of 100 mM Tris, pH 7.5.

Sephadex G-200 and Sepharose 4-B

Both chromatographic materials were obtained from Sigma. The Sepharose 4-B was "coated" with phospholipid prior to column preparation because Huang[13] found that such treatment significantly reduced adsorption of liposomes. The modified procedure that we employed involved overnight stirring at 4 °C of 3 vol. commercial Sepharose 4-B suspension with 2 vol. of a lipid dispersion containing 10 μmoles of lecithin, 7.5 μmoles of cholesterol, and 1.0 μmole of dicetyl phosphate per ml of 150 mM NaCl–50 mM Tris, pH 7.5.

Antigens, antisera and complement source

Globoside I was a generous gift of Drs S. Handa and T. Yamakawa, Faculty of Medicine, University of Tokyo; rabbit antigloboside serum was obtained by immunization with human erythrocytes as described in detail elsewhere[14]. Alkali-treated *Salmonella minnesota* mR595 lipopolysaccharide and rabbit anti-mR595 serum were the same preparations used in an earlier investigation[15]; both were kindly donated by Dr O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, Germany. Rabbit anti-Forssman serum (sheep hemolysin) was purchased from Difco Laboratories, Detroit, Mich. Prior to use, all antisera were decomplemented by heating at 56 °C for 30 min and then dialyzed against, or diluted with, cold veronal-buffered saline

(prepared according to the method of Mayer[16]) to reduce the level of endogenous glucose. For the same reason, guinea pig serum (used as the source of complement in the present experiments) was also dialyzed against Veronal-buffered saline as described previously[12].

Liposome preparation

Liposomes were generated essentially as described in previous papers[12,15] from sheep fraction IIa or from a dried lipid film containing phospholipid (lecithin or sphingomyelin), cholesterol, and charged amphiphile (dicetyl phosphate or stearylamine) in molar ratios of 2:1.5:0.2, respectively. An appropriate amount of amphipathic antigen (either globoside or alkali-treated lipopolysaccharide) was also added as dictated by the needs of the particular experiment. In all cases, the lipids were dispersed in a sufficient volume of swelling solution containing the requisite markers to yield a 10 mM phospholipid suspension.

The principal modifications of earlier procedures involved the composition of the swelling solution and the method by which untrapped markers were removed from the liposome preparation. In the present investigation, the solution in which the dried lipid film was dispersed contained not only glucose (300 mM) but also one, or a combination, of the following dialyzed enzymes: hexokinase (approx. 350 μg per ml), glucose-6-phosphate dehydrogenase (approx. 100 μg per ml), or β-galactosidase (approx. 300 μg per ml). After dispersion with the aid of a Vortex mixer, the suspension was kept at room temperature (approx. 22 °C) for 20 min followed by an additional 20 min in an ice bath. Untrapped markers were then removed from the liposome preparation by chromatography at 4 °C on columns (bed volume: approx. 1 cm × 25 cm) of either Sephadex G-200 or Sepharose 4-B. The liposome preparation (200 to 400 μl) was applied to the column and eluted with cold 150 mM NaCl–50 mM Tris, pH 7.5; the latter was the same buffer employed initially to equilibrate the columns. Fractions that contained liposomes as indicated by visual turbidity or by measurement of light scatter at 410 nm appeared within, or shortly after, the void volume. These fractions were combined to give a preparation containing approximately 0.5 to 1.0 mM phospholipid (*i.e.* a 10- to 20-fold dilution of the original suspension) for use in the experiments described below.

Experimental design

The experiments in this paper were carried out in three successive stages. The first stage involved incubation of liposomes with an appropriate "lytic agent": either phospholipase C, saponin, antiserum *plus* guinea pig serum, or filipin. The second and third stages involved, respectively, removal of liposomes from the incubation mixture by ultracentrifugation and assay of the supernatant solutions to determine the amount of marker released.

Stage 1 conditions

Except where noted the isotonic buffer mentioned in the following paragraphs refers to a pH 7.5 solution containing 150 mM NaCl, 50 mM Tris, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$; also, unless stated otherwise, all incubations were at room temperature.

In the case of liposomes prepared with lecithin as the sole phospholipid, the

total amount of markers trapped was determined after treatment with phospholipase C. The reaction mixture contained 200 μl of eluted liposome preparation, 250 μl of phospholipase C solution (250 μg of enzyme), 25 μl of 1 M $CaCl_2$ and 3.525 ml of isotonic buffer; tubes were incubated for 30 min.

In the case of liposomes prepared with either sheep fraction IIa or sphingomyelin as the sole phospholipid, phospholipase C treatment could not be used to release all the trapped markers because previous studies[17,18] have indicated that liposomes with a high content of sphingomyelin are remarkably resistant to this enzyme. Complete marker release from these liposomes was obtained by incubation of 200 μl of liposomes with 2.5 ml of water and 65 μl of saponin solution (6.5 mg of steroid). After 10 min, 500 μl of 8-fold concentrated isotonic buffer and 735 μl of water were added to give a final volume of 4 ml. In regard to the above method, reference should be made to preliminary experiments which have shown that saponin would not promote complete marker release under isotonic conditions; this difficulty was circumvented by carrying out the incubation in water.

Release of markers in the presence of antibody–complement was determined after incubation for 30 min of 200 μl of liposomes with varying amounts of the appropriate antiserum (see table and figure legends), 620 μl of guinea pig serum, and sufficient isotonic buffer to give a final volume of 4 ml.

Release of markers in the presence of filipin was determined after incubation for 15 min of 200 μl of liposomes with varying amounts of stock antibiotic solution (see table and figure legends) and sufficient isotonic buffer to bring the final volume to 4 ml. Preliminary experiments indicated that addition of the corresponding amount of solvent for the antibiotic (up to 15 μl of dimethylformamide) did not promote marker release *per se*.

Stage 2

Following Stage 1, the reaction mixtures were carefully layered over 8.1 ml of a "cushion" (300 mM sucrose–50 mM Tris, pH 7.5) contained in tubes for the Spinco SW-41 rotor. These were then centrifuged (35000 rev./min) at 20 °C for 25 min. Approx. 2.5 ml of the clear supernatant solution was removed by gentle aspiration (without disturbing the liposomes at the interface) for subsequent assay of released markers as described below.

Stage 3 conditions for glucose, hexokinase, and glucose-6-phosphate dehydrogenase

Each of these markers was assayed with a reagent (or an appropriate modification thereof) containing: 4 mM ATP, 2 mM NADP, 7 mM $MgCl_2$, 0.3 mM $CaCl_2$, 200 mM Tris (pH 7.5), 168 mM NaCl, and 35 μg of dialyzed hexokinase and 10 μg of dialyzed glucose-6-phosphate dehydrogenase per ml. Unless otherwise stated, the isotonic buffer referred to in the following paragraphs denotes a pH 7.5 solution containing 150 mM NaCl, 50 mM Tris, 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. Absorbances were measured at 340 nm in cuvettes with a 10-mm light path. Preliminary experiments were performed to establish that either the total absorbance increase (in the case of glucose) or the rate of absorbance increase (in the case of the enzymes) was directly proportional to the volume of supernatant solution assayed; the volumes specified below have routinely fulfilled these requirements.

Glucose was determined by measuring the change in absorbance that occurred

upon addition of 250 μl of the assay reagent to 750 μl of the supernatant solution; stable readings were obtained within 2–3 min. Values were corrected for dilution prior to calculation of the glucose released by antibody–complement or filipin. For this purpose, conversion of the absorbances to abolute amounts of glucose was not necessary because all results have been expressed on a percentage basis, *i.e.* relative to the amount of marker released by phospholipase C or saponin.

Similarly, the relative concentrations of the enzymes were determined from the linear rate of absorbance increase (calculated in arbitrary units of $\Delta A \cdot 10^3$ per min) under the following conditions. Cuvettes contained initially 500 μl of supernatant solution, 250 μl of assay reagent identical to the above except that the appropriate enzyme to be measured (*i.e.* hexokinase or glucose-6-phosphate dehydrogenase) was omitted, and sufficient isotonic buffer to bring the volume to 1 ml; the reaction was initiated by the addition of 6 μl of 0.3 M glucose.

Stage 3 conditions for β-galactosidase

This enzyme was assayed by measuring the initial rate of absorbance increase at 405 nm due to the hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG). Cuvettes contained 800 μl of the supernatant solution and the reaction was started by the addition of 4.3 μmoles of ONPG dissolved in 200 μl of 150 mM NaCl–50 mM Tris, pH 7.5. In the case of phospholipase C treated liposome preparations, preincubation of the supernatant aliquot for 10 min with 10 μl of 10 mM $MgCl_2$ was necessary to reverse the inhibitory effects of the $CaCl_2$ which had been added to activate the phospholipase (see Stage 1 conditions described previously).

Electron microscopy

Eluted liposomes were incubated with either filipin, or antigloboside *plus* guinea pig serum, under the Stage 1 conditions outlined above and detailed in the appropriate table and figure legends. Liposomes were recovered from the reaction mixture by centrifugation (12500 rev./min) at 20 °C for 10 min in the Spinco SW-39 rotor. The liposomal pellet was washed once with 5 ml of isotonic buffer and then dispersed by gentle agitation with a Vortex mixer in 50 μl of distilled water. In some experiments, this step was modified by the addition of 90 μl of a trypsin solution (1 mg/ml; 2 times crystallized bovine pancreas enzyme obtained from Sigma) to the buffer and incubation for 15 min prior to recentrifugation. After addition of 50 μl of a 2% phosphotungstate solution (adjusted to pH 6.5 and containing 0.0025% bovine serum albumin) to the resuspended liposomes, a drop was placed on 400 mesh copper grids previously coated with a collodion–carbon film. The grids were usually examined on the day of preparation in a Philips EM 200 electron microscope operated at 80 kV. Plates were taken at an initial instrumental magnification of 50000 with subsequent photographic enlargement as desired.

RESULTS

Validation of experimental methods

Figs 1 and 2 show that liposomes, swollen in solutions containing various combinations of markers, can be readily separated from free markers that are neither bound nor trapped by chromatography on either Sephadex G-200 or Sepharose 4-B.

It should be noted (Fig. 1) that a Sephadex G-200 column adequately removes untrapped glucose-6-phosphate dehydrogenase (mol. wt approx. 205000). For this reason, Sepharose 4-B was employed only in those experiments that involved the largest of the enzymes, *i.e.* β-galactosidase (mol. wt approx. 518000) as marker (Fig. 2).

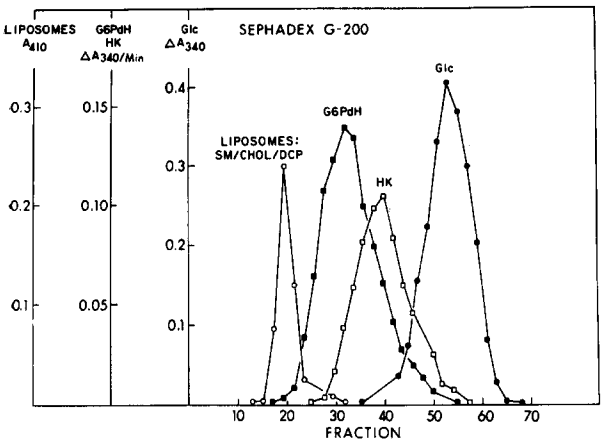

Fig. 1. Separation of sphingomyelin liposomes from untrapped markers by chromatography on Sephadex G-200. Liposomes (swollen in a solution of glucose (Glc), hexokinase (Hk), and glucose-6-phosphate dehydrogenase (G6PdH) were prepared from a mixture of sphingomyelin–cholesterol–dicetyl phosphate (SM/Chol/DCP) and chromatogrammed on a Sephadex G-200 column as described in Methods. Appropriate aliquots of the eluted fractions (approx. 260 μl per tube) were then assayed for the presence of untrapped markers using the same "Stage 3" conditions that were employed in subsequent experiments for measuring the release of trapped markers. The presence of liposomes was detected by the increased light scatter at 410 nm (ref. 19). Identical elution profiles were obtained with liposomes prepared from lecithin and when antigen was incorporated.

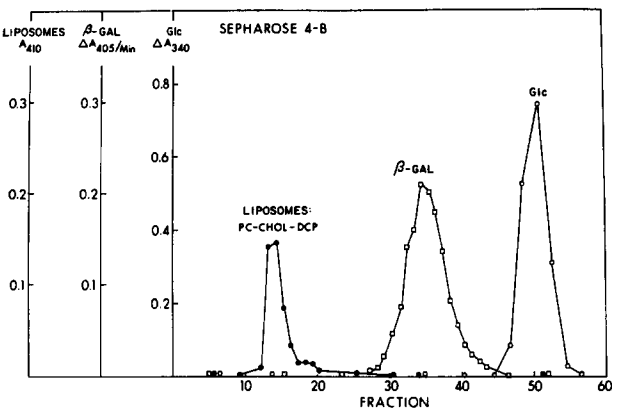

Fig. 2. Separation of lecithin liposomes from untrapped markers by chromatography on Sepharose 4-B. Liposomes (swollen in a solution of glucose (Glc) and β-galactosidase (β-Gal)) were prepared from a mixture of phosphatidylcholine–cholesterol–dicetyl phosphate (PC–Chol–DCP). Procedure otherwise similar to that described in the legend to Fig. 1 except that a Sepharose 4-B column was employed and each fraction contained approx. 550 μl per tube. Identical elution profiles were obtained with liposomes prepared from sphingomyelin and when antigen was incroporated.

Assay of the supernatant solutions obtained after treatment with either phospholipase C or saponin indicates, however, that the enzymes (and glucose) are present in the fractions which contain the eluted liposomes (Table I). Sessa and Weissmann[19] have emphasized that at least two criteria must be met before it can be concluded that an enzyme associated with liposomes is actually trapped in the aqueous regions. First, the amount of marker present should show a positive correlation with the size of the aqueous compartments within the liposomes. This condition was satisfied by the finding that less of each of the markers was associated with the liposomes when they were swollen in solutions of increasing ionic strength, *i.e.* increasing concentrations of NaCl (Table I). Previous theoretical and experimental considerations[20] have demonstrated that liposomal volume is inversely related to the ionic strength of the media in which the lipids are dispersed.

TABLE I

EFFECT OF IONIC STRENGTH ON MARKER TRAPPING BY LECITHIN LIPOSOMES

Phosphatidylcholine–cholesterol–dicetyl phosphate liposomes were prepared by a modification of the standard procedure in which the swelling solutions contained fixed concentrations of glucose-6-phosphate dehydrogenase and β-galactosidase, but varying concentrations of glucose, as markers. Constant tonicity was maintained by the addition of appropriate concentrations of NaCl (see table) which also produced the desired variation in ionic strength of the swelling solution. During Stage 1, 200 μl of liposomes (eluted from Sepharose 4-B) were incubated with either phospholipase C or saponin as specified in Methods; the eluted liposomes were also assayed for their content of total phosphate. For the enzyme markers, results are expressed as $\Delta A_{340\ nm}$ per min per nmole phospholipid $\times 10^{-3}$ (glucose-6-phosphate dehydrogenase) and $\Delta A_{405\ nm}$ per min per nmole phospholipid $\times 10^{-3}$ (β-galactosidase). For glucose, results are expressed as nmoles per nmole phospholipid after correction for the difference in initial marker concentration. For example, in the case where the swelling solution contained 100 mM glucose, the actual experimental values were multiplied by 3 to permit comparison with the swelling solution in which the glucose concentration was 300 mM. Glc, glucose; G6PdH, glucose-6-phosphate dehydrogenase; β-Gal, β-galactosidase.

Swelling solution	*Liposomes incubated with*	*Specific trapping of*		
		Glc	*G6PdH*	*β-Gal*
300 mM Glc	Phospholipase C	2.54	8.03	1.58
	Saponin	2.84	7.80	1.84
200 mM Glc–	Phospholipase C	1.34	5.53	0.77
50 mM NaCl	Saponin	1.43	5.52	0.77
100 mM Glc–	Phospholipase C	0.48	3.15	0.58
100 mM NaCl	Saponin	0.50	3.35	0.62

The enzymes employed as markers all bear a net negative charge at the pH (pH 7.5) used in this study. Table II records the results of experiments in which "preformed" lecithin or sphingomyelin liposomes (swollen in glucose only) were subsequently incubated in solutions containing different combinations of enzymes prior to column chromatography. The eluted negatively charged liposomes (prepared with dicetyl phosphate) bound negligible amounts of the enzymes when compared with positively charged liposomes (prepared with stearylamine). This was consistent

TABLE II

ENZYME BINDING TO ANIONIC AND CATIONIC LECITHIN OR SPHINGOMYELIN LIPOSOMES

Liposomes (swollen in a solution of glucose) were prepared from the lipid mixtures whose composition is listed below and maintained at room temperature for 2 h. β-Galactosidase (β-Gal) and glucose-6-phosphate dehydrogenase (G6PdH) or hexokinase (HK) (see table) were then added to the liposome suspensions at the final concentrations present in the standard swelling solution. After 5 min, the incubation mixtures were chromatogrammed on Sepharose 4-B columns. Eluted liposomes were assayed for the presence of the enzyme markers after incubation with either phospholipase C or saponin in the experimental cuvettes; in control cuvettes, neither lytic agent was added. The amount of enzyme bound per nmole of liposomal phospholipid is expressed in the same units specified in the legend to Table I. PC, phosphatidylcholine; Chol, cholesterol; DCP, dicetyl phosphate; SM, sphingomyelin; SA, stearylamine.

Liposome composition	*Enzyme marker added*	*Marker present after incubation with:*		
		No agent	*Phospholipase C*	*Saponin*
PC–Chol–	G6PdH	0.8	0.8	—
DCP	β-Gal	0.02	0.05	—
SM–Chol–	HK	0	—	0.03
DCP	β-Gal	0	—	0.007
PC–Chol–	G6PdH	14.7	20.6	—
SA	β-Gal	9.3	10.1	—
SM–Chol–	HK	3.3	—	4.6
SA	β-Gal	3.3	—	3.4

with visual observations because addition of the enzymes to the cationic liposomes produced considerable clumping whereas no effect was apparent with the anionic liposomes. Furthermore, it should be noted that enzyme activity measured after treatment of the various liposomes with either phospholipase C or saponin was essentially identical to that obtained when these agents were omitted. Only negatively charged liposomes were used in the experiments described below and, therefore, these results fulfill the second of the criteria stressed by Sessa and Weissmann[19]: namely, exclusion of the possiblity that enzymes associated with liposomes swollen in their presence are not trapped but electrostatically bound to the bilayers in such a manner that their activity is masked.

Finally, it should be emphasized that phospholipase C releases the same amount of glucose and enzyme activity as does saponin (Table I). These results indicate that the levels determined after incubation of lecithin liposomes with phospholipase C (or sphingomyelin liposomes with saponin) provide a reliable measure of the amount of each marker trapped within the liposomes.

Immune release of markers from lecithin liposomes actively sensitized with globoside

Table III shows that macromolecular markers are released from lecithin liposomes, prepared in the presence of globoside, after incubation with both antigloboside serum and native guinea pig serum. Three lines of evidence indicate that release of the enzymes is a consequence of specific antibody–complement interaction with the model membrane. First, antigloboside serum has little effect in the presence of guinea pig

TABLE III

IMMUNE RELEASE OF MARKERS FROM LECITHIN LIPOSOMES ACTIVELY SENSITIZED WITH GLOBOSIDE

Liposomes (swollen in a solution of glucose (Glc), glucose-6-phosphate dehydrogenase (G6PdH) and β-galactosidase (β-Gal)) were prepared from mixtures of phosphatidylcholine–cholesterol–dicetyl phosphate that had been supplemented with the amounts of globoside indicated below. During Stage 1, liposomes (eluted from Sepharose 4-B) were incubated with native or heated guinea pig serum *plus* 113 μl of antigloboside serum or 62 μl of diluted (1:10) anti-Forssman-serum. Results are expressed relative to the amount of marker released when the liposomes were incubated with phospholipase C. PC, phosphatidylcholine.

Globoside incorporated (μg/μmole PC)	*Marker*	*Percent marker released in presence of*		
		Anti-globoside plus		*Anti-Forssman plus native guinea pig serum*
		Native guinea pig serum	*Heated guinea pig serum*	
20	Glc	50.1	0	0
20	G6PdH	59.0	0	0
20	β-Gal	51.7	3.6	3.6
4	Glc	18.5	0	0
4	G6PdH	13.9	3.6	3.6
4	β-Gal	32.9	1.9	1.9
0	Glc	0.9	0	0
0	G6PdH	2.0	0.4	0.4
0	β-Gal	7.0	1.5	1.5

serum that had been heated at 56 °C for 30 min to destroy hemolytic complement activity. Second, antigloboside serum cannot be replaced by anti-Forssman serum consistent with the fact that Forssman and globoside are ceramide antigens which possess different immunological determinants[21]. Third, negligible enzymatic activity is released after incubation with antigloboside serum and guinea pig serum from lecithin liposomes that do not contain any globoside.

Of particular significance is the finding that, within experimental error, the percentage release of the enzymes parallels the percentage of glucose loss under a variety of conditions. For example, reduction in the amount of globoside incorporated into the lipid mixture from 20 μg/μmole lecithin to 4 μg/μmole lecithin produces liposomes that release less of each of the markers examined (Table III). This aspect will be considered in greater detail below.

Immune release of markers from lecithin liposomes passively sensitized with alkali-treated lipopolysaccharide

Previous studies[12] have shown that ceramide antigens, such as globoside, must be present at the time liposomes are generated in order to confer immune sensitivity onto the model membrane; this has been designated active sensitization of liposomes. Alkali-treated lipopolysaccharides and lipid A are, however, able to passively sensitize liposomes to the action of antibody–complement after the model membrane had been formed[15,22]. Table IV demonstrates that such passively sensitized lecithin liposomes also release enzyme markers in parallel with glucose after incubation with the ap-

TABLE IV

IMMUNE RELEASE OF MARKERS FROM LECITHIN LIPOSOMES PASSIVELY SENSITIZED WITH LIPOPOLYSACCHARIDE

Liposomes (swollen in a solution of glucose (Glc), glucose-6-phosphate dehydrogenase (G6PdH), and β-galactosidase (β-Gal)) were prepared from a mixture of phosphatidylcholine–cholesterol–dicetyl phosphate that did not contain any antigen. During Stage 1, 200 μl of liposomes (eluted from Sepharose 4-B) were preincubated for 10 min with 5.25 μg of alkali-treated mR595 lipopolysaccharide before the addition of 620 μl of native or heated guinea pig serum *plus* 68 μl of anti-mR595 antiserum. Results are expressed relative to the amount of marker released by phospholipase C.

Component omitted or added during Stage 1	*Percent marker released*		
	Glc	*G6PdH*	*β-Gal*
None	51.3	41.5	58.2
−native guinea pig serum, +heated guinea pig serum	0	1.1	0
−anti mR595 antiserum	1.6	2.1	3.0
−mR595 (alkali-treated)	1.4	1.1	3.0

propriate antiserum and guinea pig serum. Negligible loss of marker was observed when native guinea pig serum was replaced by heated guinea pig serum, and when antiserum or the lipopolysaccharide antigen were omitted.

Immune release of markers from sphingomyelin liposomes actively sensitized with globoside

Analogous experiments performed with sphingomyelin liposomes are summarized in Table V. These liposomes, when prepared in the presence of globoside, also released macromolecular markers in a reaction that was dependent on the presence of both antiserum and unheated guinea pig serum. However, in contrast to the results obtained with lecithin liposomes, the percentage of enzyme activity released was significantly smaller and did not parallel the extent of glucose release (Expt a).

Several modifications of the standard Stage 1 conditions were tested in an attempt to produce a greater release of the enzyme markers from sphingomyelin liposomes. Previous studies[12,14] have indicated that the extent of glucose loss is determined by the number of antigen–antibody complexes within the liposomal bilayer; the latter can be increased by incorporation of more antigen into the lipid mixture and/or incubation with greater concentrations of antiserum. Liposomes prepared with high levels of globoside (42 μg/μmole sphingomyelin) did not, however, release any (more enzyme activity even in the presence of an amount of anti-globoside serum (228 μl) that was at least 4 times greater than that required for maximum glucose release (Expt b). In addition, there was no change in the amount of enzyme released when the time of incubation was increased from 30 to 120 min, and when the temperature was raised from 22 °C to 39 °C (compare Expt c with Expt a); the higher temperature was tested on the assumption that a greater fluidity on the part of the phospholipid paraffinic chains might facilitate loss of the macromolecular marker from the liposomes.

TABLE V

IMMUNE RELEASE OF MARKERS FROM SPHINGOMYELIN LIPOSOMES ACTIVELY SENSITIZED WITH GLOBOSIDE

Liposomes (swollen in a solution of glucose (Glc), hexokinase (HK), and β-galactosidase (β-Gal)) were prepared from mixtures of sphingomyelin–cholesterol–dicetyl phosphate that had been supplemented with the amounts of globoside indicated below. During Stage 1, 200 μl of liposomes (eluted from Sepharose 4-B) were incubated with 620 μl of native or heated guinea pig serum *plus* the volume of antiglobosido serum specified in the table. Results are expressed relative to the amount of marker released by saponin. In the case of Expt b, it should be noted that the percentage of both non-immune and immune glucose release (absence and presence of antiserum, respectively) was unusually high compared to the loss of glucose marker that was normally observed (see Expts a and c).

Globoside incorporated (μg/μmole SM)	*Antiglobosido serum added (μl)*	*Incubation conditions during Stage 1*	*Percent marker released*		
			Glc	*HK*	*β-Gal*
Expt a					
0	57	30 min, 22 °C	2.0	0	0
20	57	30 min, 22 °C	43.9 (0)*	13.0 (3.2)*	17.7 (4.5)*
42	57	30 min, 22 °C	57.5	11.1	10.7
Expt b					
42	0	30 min, 22 °C	11.1	3.2	3.7
42	57	30 min, 22 °C	76.3	14.0	—
42	114	30 min, 22 °C	81.7	12.9	3.9
42	228	30 min, 22 °C	77.6	12.3	3.4
Expt c					
20	57	120 min, 22 °C	46.8	—	11.7
20	57	120 min, 39 °C	48.0 (0)*	—	10.6 (6.4)*

* Values in parentheses indicate the percent marker released when heated guinea pig serum was substituted for native guinea pig serum.

TABLE VI

IMMUNE RELEASE OF MARKERS FROM SHEEP IIa LIPOSOMES

Liposomes (swollen in a solution of glucose (Glc), hexokinase (HK), and β-galactosidase (β-Gal)) were prepared from sheep fraction IIa. During Stage 1, 200 μl of liposomes (eluted from Sepharose 4-B) were incubated with 620 μl of native or heated guinea pig serum *plus* 62 μl of diluted (1:10) anti-Forssman serum. Results are expressed relative to the amount of marker released by saponin.

Marker	*Percent marker released by anti-Forssman plus*	
	Native guinea pig serum	*Heated guinea pig serum*
Glc	48.5	6.4
HK	13.2	1.2
β-Gal	9.0	3.7

Immune release of markers from sheep fraction IIa liposomes

Because the formation and properties of the characteristic complement-induced lesions have been most extensively investigated with sheep erythrocytes, the release of enzymes from liposomes prepared with the lipids isolated from these cells (sheep fraction IIa) has also been examined. Table VI shows that such liposomes release macromolecules when incubated with anti-Forssman serum and native (but not heated) guinea pig serum. It is important to note, however, that the percentage release of the enzymes is very low and does not parallel that of glucose. The sheep IIa liposomes thus resemble sphingomyelin, rather than lecithin, liposomes in their response to antibody–complement. This finding is entirely consistent with the fact that nearly 50% of the sheep erythrocyte phospholipid is sphingomyelin and lecithin is present, if at all, in trace amounts[5].

Release of markers from liposomes treated with filipin

In connection with the subsequent electron microscopic experiments, it was considered essential to examine the effect of this polyene antibiotic on lecithin and sphingomyelin liposomes. Table VII shows that incubation with filipin results in a considerable release of enzymatic activity from liposomes prepared with either phospholipid. Moreover, the percentage release of macromolecular marker paralleled the percentage release of glucose; both were dependent on the initial concentration of antibiotic. The response of sphingomyelin liposomes to the polyene thus contrasts markedly with their response to antibody–complement as discussed above.

TABLE VII

RELEASE OF MARKERS FROM LECITHIN AND SPHINGOMYELIN LIPOSOMES BY FILIPIN

Liposomes (swollen in a solution of glucose (Glc) and hexokinase (HK) or glucose-6-phosphate dehydrogenase (G6PdH)) were prepared from mixtures of phosphatidylcholine–cholesterol–dicetyl phosphate (PC–Chol–DCP) or sphingomyelin–cholesterol–dicetyl phosphate (SM–Chol–DCP) as indicated below. During Stage 1, 200 μl of liposomes (eluted from Sephadex G-200) were incubated with the amounts of filipin specified in the table. Results are expressed relative to the amount of marker released by phospholipase C (lecithin liposomes) or saponin (sphingomyelin liposomes).

Liposome composition	*Filipin present during Stage 1* (μg)	*Percent marker released*		
		Glc	*HK*	*G6PdH*
PC–Chol–DCP	28	27.4	—	31.3
	122	90.4	—	97.0
SM–Chol–DCP	28	38.5	22.5	—
	122	91.4	57.0	—

Electron microscopic observations

The preceding experiments led to the formulation of the following working hypothesis (*cf.* Introduction). Assuming that the lesions are actual holes in the membrane through which the macromolecules escape, they should be present in sensitized

lecithin liposomes after treatment with antibody and complement. Furthermore, if the lesions are functional holes, few (if any) should be found in sphingomyelin liposomes incubated under similar conditons because these liposomes release very little of the enzyme markers. This hypothesis did not withstand experimental testing; no clear evidence for the presence of lesions in either the lecithin or sphingomyelin liposomes was obtained. The pictures described below refer only to observations made on lecithin liposomes actively sensitized with globoside because lesions were anticipated in these model membranes. It should be emphasized, however, that experiments with liposomes prepared from sphingomyelin gave entirely identical results.

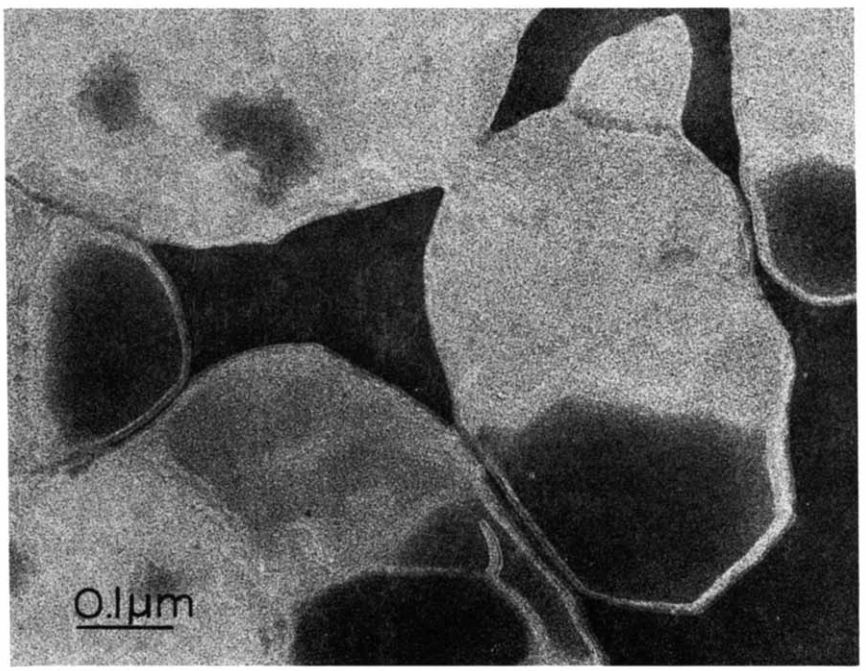

Fig. 3. Electron micrograph of lecithin liposomes, actively sensitized with globoside, incubated in the absence of antiserum and guinea pig serum. Liposomes (swollen in a solution of glucose and hexokinase or glucose-6-phosphate dehydrogenase) were prepared from a mixture of phosphatidylcholine–cholesterol–dicetyl phosphate containing 20 μg globoside per μmole phosphatidylcholine. During Stage 1, 200 μl of liposomes (eluted from Sephadex G-200) were incubated in isotonic buffer alone.

Fig. 3 is a picture of control liposomes incubated alone during Stage 1, *i.e.* in the absence of antiserum and guinea pig serum. This electron micrograph should be contrasted with Fig. 4 showing liposomes that had been incubated with both antiserum and native guinea pig serum; in the latter, "fuzzy" material is apparent at the edges, and on the surface, of the liposomes. As shown in Fig. 5, this material is not present in liposomes incubated with antiserum and heated guinea pig serum and therefore probably represents a product of complement activation. Fig. 6 suggests that this material is predominately protein because it is almost completely absent in liposome

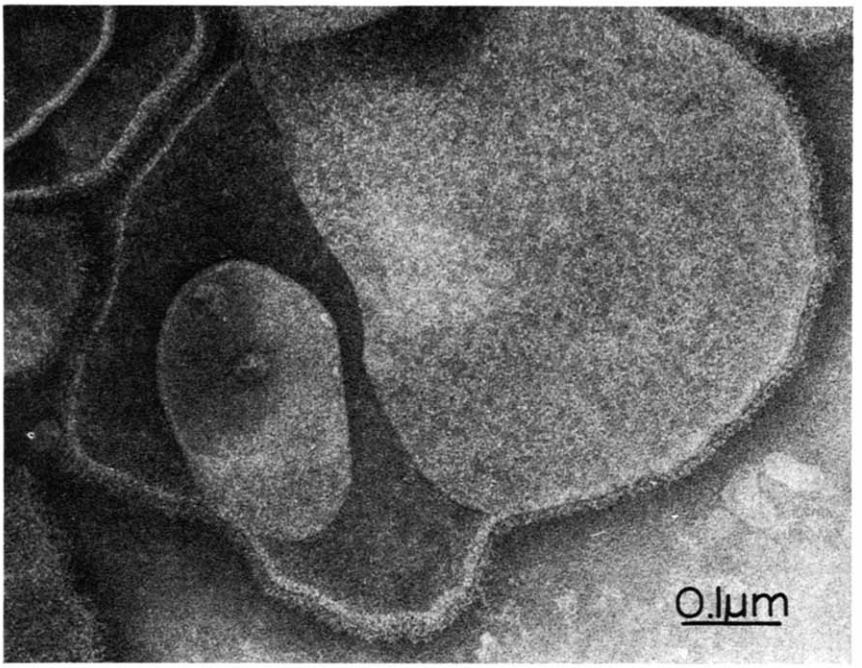

Fig. 4. Electron micrograph of lecithin liposomes, actively sensitized with globoside, incubated in the presence of antiserum and native guinea pig serum. Conditions similar to those described in the legend to Fig. 3 except that, during Stage 1, liposomes were incubated in the presence of 113 μl of antigloboside serum and 620 μl of native guinea pig serum.

preparations that had been treated with trypsin after incubation with both antiserum and native guinea pig serum. These findings support and extend previous studies[23] which have demonstrated that sensitized liposomes, in the presence of an appropriate antiserum, can bind appreciable amounts of protein derived from native (but not heated) guinea pig or human serum.

The preceding electron micrographs are representative of numerous experiments and failure to observe the characteristic lesions has been our consistent experience. A rare example of what might perhaps be considered as lesions ("rings") is shown in Fig. 7; this was observed in a liposome preparation that was first incubated with antiserum and native guinea pig serum, and then subjected to trypsin treatment. In this regard, reference should be made to the original observations of Humphrey and Dourmashkin[2], and confirmed by Polley *et al.*[3], that lesions were more easily visualized in complement damaged erythrocyte membranes after proteolysis with trypsin. These findings suggested that the inability to detect lesions in liposomes treated with antibody–complement may have been due to "masking" by the bound protein and prompted the experiment described in Fig. 7. It should, however, be emphasized that such pictures were infrequently obtained from a limited number of plates in some, but not all, experiments and cannot be regarded as typical (*cf.* Fig. 6). Perhaps of even greater significance is the fact that the presence of bound protein did not interfere with the visualization of lesions produced by filipin. This is demonstrated by Fig. 8

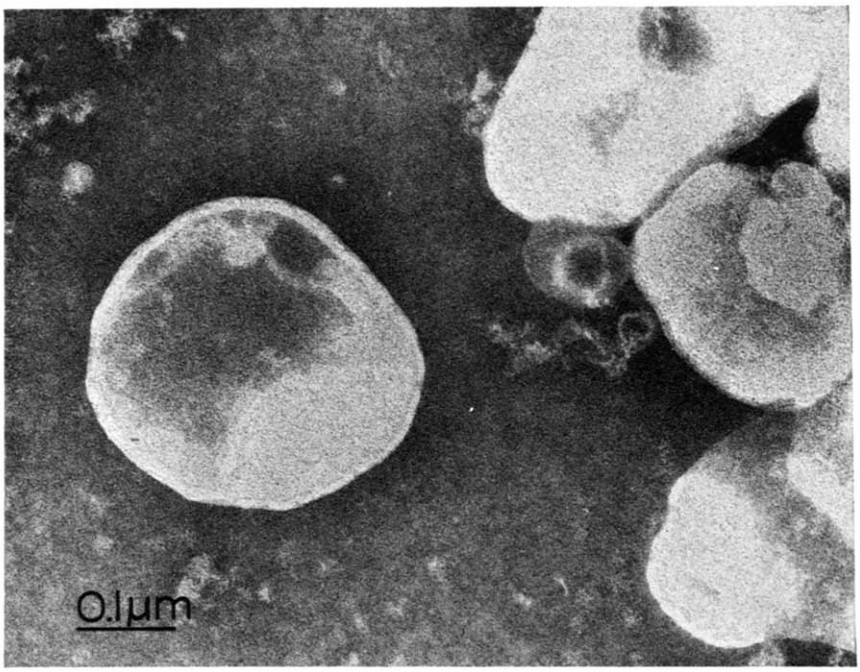

Fig. 5. Electron micrograph of lecithin liposomes, actively sensitized with globoside, incubated in the presence of antiserum and heated guinea pig serum. Conditions similar to those described in the legend to Fig. 3 except that, during Stage 1, liposomes were incubated in the presence of 113 μl of antigloboside serum and 620 μl of heated guinea pig serum.

showing sensitized liposomes that had been incubated with both the antibiotic and antiserum *plus* native guinea pig serum. Under such conditions, the polyene-induced lesions were consistently observed.

DISCUSSION

The feasibility of using trapped enzymes as macromolecular markers for following changes in liposomal permeability was initially suggested by the report of Sessa and Weissmann[19] who demonstrated the incorporation of egg white lysozyme into the aqueous compartments of liposomes. However, for the purpose of this investigation (see Introduction), we originally considered lysozyme of mol. wt approx. 16000 as too small and, accordingly, employed larger enzymes whose concentration can be readily determined by spectrophotometric assays. In this connection, it should be noted that yeast hexokinase[24,25], yeast glucose-6-phosphate dehydrogenase, and *E. coli* β-galactosidase[26] exist predominately as multimeric forms which have mol. wts of 102000 205000, and 518000, respectively.* In addition, β-galactosidase has been extensively

* Yue *et al.*[27] have reported that the molecular weight of NADP-free yeast glucose-6-phosphate dehydrogenase is approx. 102400. However, the glucose-6-phosphate dehydrogenase preparations used in this study behaved chromatographically similar to proteins with a molecular weight around 200000. This was probably due to the presence of bound NADP because Yoshida[28] has shown that removal of the coenzyme from human erythrocyte glucose-6-phosphate dehydrogenase (mol. wt approx. 240000) causes dissociation into subunits with a molecular weight of about one-half of the native protein.

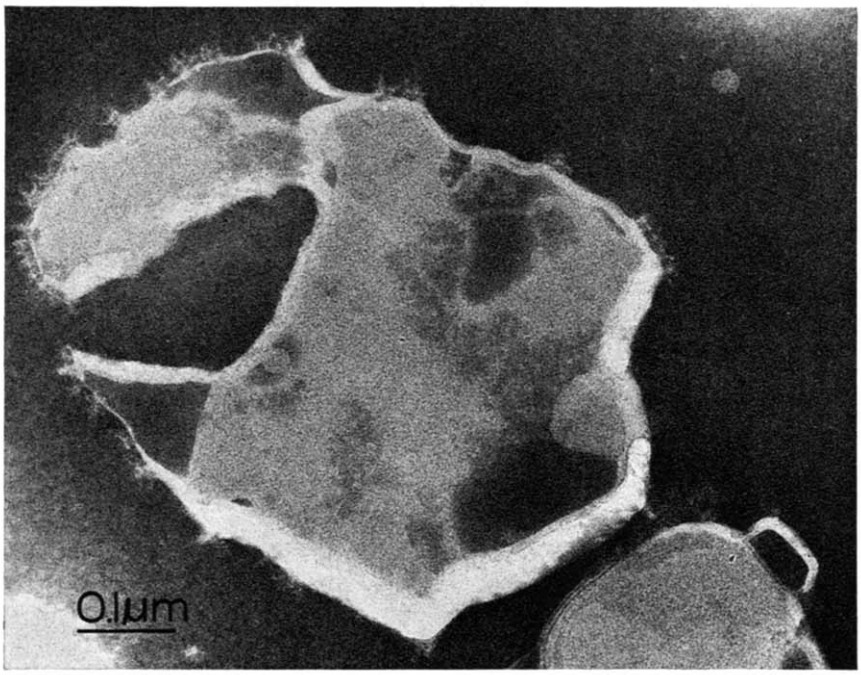

Fig. 6. Electron micrograph of trypsin-treated lecithin liposomes, actively sensitized with globoside, incubated in the presence of antiserum and native guinea pig serum. Conditions similar to those described in the legend to Fig. 3 except that, during Stage 1, liposomes were incubated in the presence of 113 μl of antigloboside serum and 620 μl of native guinea pig serum; the reaction mixture was then incubated with trypsin as described in Methods.

investigated with the electron microscope and these studies[26] indicate that the enzyme exists mainly as quadrangular particles (squares or rhombuses). Indeed, one of the reasons for employing β-galactosidase as marker was that two sides are of equal length (115 Å) which closely approximate the diameter of the lesions found in natural membranes after complement dependent cytolysis.*

Thus, if the complement dependent loss of enzyme marker from liposomes proceeds through holes that are of the anticipated dimensions and stable to the experimental procedures employed (see below), they should have been detected in the electron microscope. Micrographs were particularly examined for the characteristic lesions described by Humphrey and Dourmashkin[2] but with little success; as noted above, only on a very few occasions have "ring" forms been detected. Our observations on liposomes therefore confirm those of Knudsen *et al.*[8] and are consistent with the suggestion of Polley and co-workers[3,4] that lesions may not represent discrete holes.

The previous inability of Knudsen *et al.*[8] to find any lesions was attributed by Hesketh *et al.*[9] to a possible reassembly of damaged liposomes into smaller fragments.

---

* We are indebted to Dr Carl Frieden, Department of Biochemistry, Washington University School of Medicine, for suggesting the use of β-galactosidase in view of these properties.

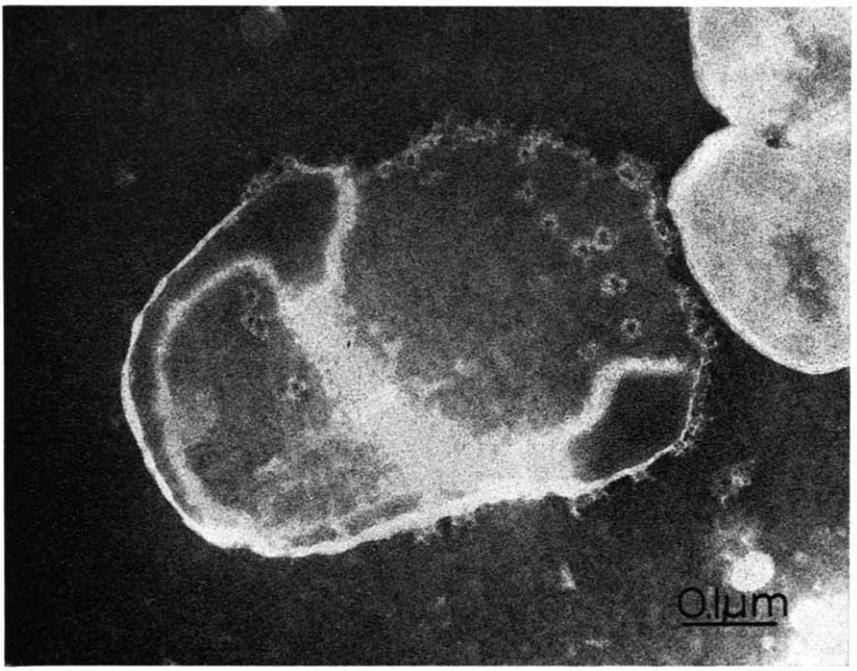

Fig. 7. Electron micrograph of trypsin-treated lecithin liposomes, actively sensitized with globoside, incubated in the presence of antiserum and native guinea pig serum. See legend to Fig. 6.

The present work has shown, however, that filipin also damages liposomes to an extent which permits the release of enzyme markers and that such lipsomes consistently manifest the characteristic lesions produced by the antibiotic. This difference between liposomes treated with antibody–complement and those treated with filipin again suggests that the lesions produced by the latter are actual holes through the membrane[10]. Evidence in favor of this hypothesis has been obtained previously by examining the effects of various osmotic stabilizers on polyene-induced hemolysis of rat erythrocytes (reviewed in ref. 29). On the other hand, it is recognized that the correlation between the efficacy of various compounds in preventing hemolysis by different polyene antibiotics and the presence or absence of lesions in the erythrocyte membrane may be entirely circumstantial. Thus, the apparent dimensions of the functional hole revealed by osmotic stabilization experiments could merely reflect transient pertubations in membrane structure (for example, a rapid and reversible lamellar to micellar phase transition) that is not "fixed" by the negative stain. Structural changes produced by either the polyene antibiotics or complement might perhaps be revealed by alternative electron microscopic procedures involving freeze etching or fracturing*.

* Preliminary experiments utilizing this approach were discussed by Seeman at a recent symposium[31]. "Pits", which presumably correspond to the negatively stained lesions, were detected on the exterior etch-face of sheep erythrocyte membranes after immune lysis; they were rarely present on the interior etch-face suggesting that the pits do not extend through the membrane as a hole.

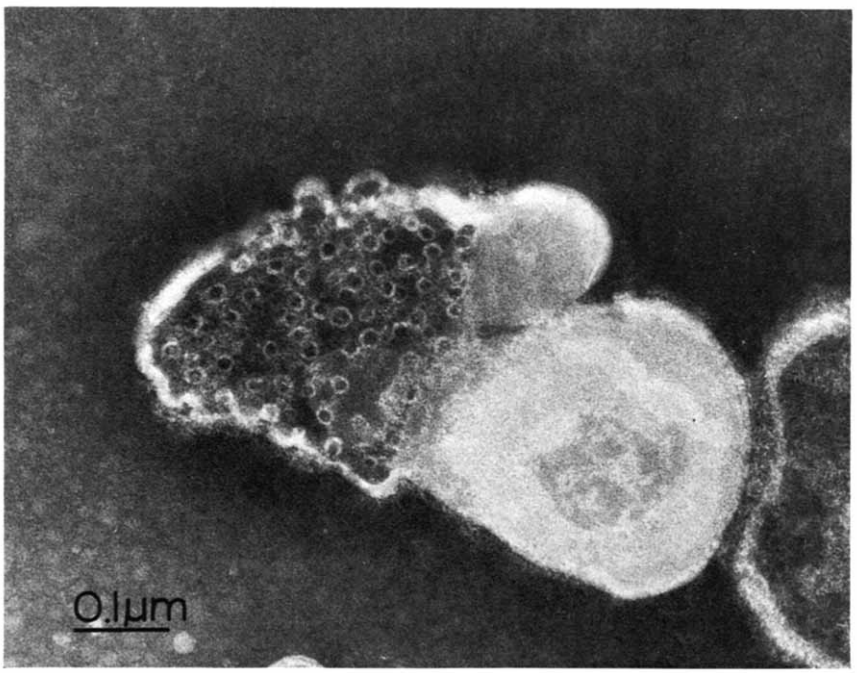

Fig. 8. Electron micrograph of lecithin liposomes, actively sensitized with globoside, incubated in the presence of filipin, antiserum, and native guinea pig serum. Conditions similar to those described in the legend to Fig. 3 except that, during Stage 1, liposomes were incubated (15 min) with 28 μg of filipin; 113 μl of antiserum and 620 μl of native guinea pig serum were then added and incubation continued for another 15 min.

Our caution in equating the filipin-induced lesions with passages through the membrane is due in large measure to the accumulating evidence that the lesions produced by complement may not be holes (see footnote preceding page). Polley and co-workers[3,4] have nevertheless shown that the lesions, which appear after the reaction of C5, reflect a structural alteration of the erythrocyte membrane and not accretion of complement proteins on the surface because they persist after removal of C3 and C5 by trypsin digestion. As a consequence, they have suggested that the lesions may represent preselected sites for the subsequent action of the terminal complement components (for example, C8) necessary to produce hemolysis. In this connection, it is of course puzzling that Lachmann *et al.*[7] were not able to visualize any lesions after the reaction of liposomes with C5 but did when C8 and C9 were subsequently added: this could have been due to a relatively low initial input of C5 on the assumption that several C5 molecules in close proximity are required for the formation of lesions. A requirement for "neighboring" C5 molecules may also explain why Hesketh *et al.*[9] have been able to detect lesions in complement treated liposome preparations. We suggest, as a possible explanation, that in such cases the sensitizing antigen may not have been uniformally distributed in the lipid bilayers: localized regions with a high concentration of antigen would be expected to bind more antibody resulting in a fixation of greater amounts of C5.

Finally, it should be noted that observations made during the course of this

investigation also have some bearing on the mechanism by which complement damages these multicompartment liposomes. In this regard, it seems significant that, upon incubation with excess antibody and complement, liposomes prepared with sphingomyelin (or sheep IIa) release a far smaller percentage of the enzyme markers than those prepared with lecithin. The amount of glucose lost from the two types of liposomes is, however, comparable and corresponds to approx. 50–80% of the glucose that is trapped. Previous calculations[6] indicate that the latter value is appreciably greater than the quantity of glucose sequestered in the outermost compartment (approx. 10% of the amount trapped) and, accordingly, some of the released glucose presumably originates from the "deeper" aqueous compartments. As a possible mechanism for the latter, we have proposed[6,17,30] that activation of the complement component responsible for membrane damage results in the exposure of hydrophobic regions in this protein (or liberation of fragments containing such regions) which then diffuse to the internal bilayers. In view of the above results showing that an enzyme of mol. wt 102000 (hexokinase) is not readily released from complement damaged sphingomyelin liposomes, it seems likely that a protein of similar size would experience difficulty in penetrating the interior of such liposomes. Experiments are now in progress to determine whether proteins of lower molecular weights are released in parallel with glucose after immune damage of both sphingomyelin and lecithin liposomes.

ACKNOWLEDGEMENTS

This research was supported by U.S.P.H.S. Grant No. AI-09319 (to S.C.K.) and Grant No. HE-13694 (to J. R.W.).

REFERENCES

1 Borsos, T., Dourmashkin, R. R. and Humphrey, J. H. (1964) *Nature* 202, 251
2 Humphrey, J. H. and Dourmashkin, R. R. (1969) *Adv. Immunol.* 11, 75
3 Polley, M. J., Müller-Eberhard, H. J. and Feldman, J. D. (1971) *J. Exp. Med.* 133, 53
4 Polley, M. J. (1971) in *Progress in Immunology* (Amos, B., ed.), p. 596, Academic Press, New York
5 Haxby, J. A., Kinsky, C. B. and Kinsky, S. C. (1968) *Proc. Natl. Acad. Sci. U.S.* 61, 300
6 Kinsky, S. C. (1972) *Biochim. Biophys. Acta*, 265, 1
7 Lachmann, P. J., Munn, E. A. and Weissmann, G. (1970) *Immunology* 19, 983
8 Knudsen, K. C., Bing, D. H. and Kater, L. (1971) *J. Immunol.* 106, 258
9 Hesketh, T. R., Dourmashkin, R. R., Payne, S. N., Humphrey, J. H. and Lachmann, P. J. (1971) *Nature* 233, 620
10 Kinsky, S. C., Luse, S. A., Zopf, D., van Deenen, L. L. M. and Haxby, J. (1967) *Biochim. Biophys. Acta* 135, 844
11 Kataoka, T. and Kinsky, S. C. (1971) *Fed. Proc.* 30, 355
12 Kinsky, S. C., Haxby, J. A., Zopf, D. A., Alving, C. R. and Kinsky, C. B. (1969) *Biochemistry* 8, 4149
13 Huang, C. (1969), *Biochemistry* 8, 344
14 Inoue, K., Kataoka, T. and Kinsky, S. C. (1971) *Biochemistry* 10, 2574
15 Kataoka, T., Inoue, K., Lüderitz, O. and Kinsky, S. C. (1971) *Eur. J. Biochem.* 21, 80
16 Mayer, M. M. (1961) in *Experimental Immunochemistry* (Kabat, E. A. and Mayer, M. M., eds), p. 133, C. C. Thomas, Springfield, Ill.
17 Inoue, K. and Kinsky, S. C. (1970) *Biochemistry* 9, 4767
18 Zwaal, R. F. A., Roelofsen, B., Comfurius, P. and van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* 233, 474

19 Sessa, G. and Weissmann, G. (1970) *J. Biol. Chem.* 245, 3295
20 Bangham, A. D., De Gier, J. and Greville, G. D. (1967) *Chem. Phys. Lipids* 1, 225
21 Siddiqui, B. and Hakomori, S. (1971) *J. Biol. Chem.* 246, 5766
22 Kataoka, T., Inoue, K., Galanos, C. and Kinsky, S. C. (1971) *Eur. J. Biochem.* 24, 123
23 Alving, C. R. and Kinsky, S. C. (1971) *Immunochemistry* 8, 325
24 Pringle, J. R. (1970) *Biochem. Biophys. Res. Commun.* 39, 46
25 Schmidt, J. J. and Colowick, S. P. (1970) *Fed. Proc.* 29, 334
26 Karlsson, U., Koorajian, S., Zabin, I., Sjöstrand, F. S. and Miller, A. (1964) *J. Ultrastruct. Res.* 10, 457
27 Yue, R. H., Roy, R. N., Noltmann, E. A. and Kuby, S. A. (1966) *Fed. Proc.* 25, 712
28 Yoshida, A. (1966) *J. Biol. Chem.* 241, 4966
29 Kinsky, S. C. (1970) *Annu. Rev. Pharmacol.* 10, 119
30 Uemura, K. and Kinsky, S. C. (1972) *Biochemistry* 11, 4085
31 Seeman, P. (1972) *Chem. Phys. Lipids* 8, 270