Active *vs.* Passive Sensitization of Liposomes toward Antibody and Complement by Dinitrophenylated Derivatives of Phosphatidylethanolamine†

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ABSTRACT: Dinitrophenylated derivatives of phosphatidylethanolamine, lysophosphatidylethanolamine, and glycerophosphorylethanolamine have been synthesized for potential use as antigens in the preparation of immunologically responsive liposomal model membranes. These compounds were tested under conditions of active sensitization (antigen present at the time the model membrane was generated) and passive sensitization (antigen added after formation of liposomes). Both dinitrophenylated phosphatidylethanolamine and lysophosphatidylethanolamine were capable of actively sensitizing liposomes toward antibody–complement, whereas only the latter could passively sensitize liposomes. Release of trapped glucose marker from either actively or passively sensitized liposomes required the presence of both anti-dinitrophenyl antibodies and native guinea pig serum as source of complement. High-affinity antibodies (as determined by fluorescence quenching with ϵ-dinitrophenyllysine) were bound to sensitized liposomes to a greater extent, and were more effective in promoting loss of marker, than low-affinity antibodies. Dinitrophenylated glycerophosphorylethanolamine did not function in either the active or passive sensitization procedures but did act as a water-soluble hapten that inhibited glucose release from actively sensitized liposomes. Passively sensitized liposomes, freed from unincorporated dinitrophenylated lysophosphatidylethanolamine by Sephadex chromatography, released as much marker as actively sensitized liposomes. This observation may provide an important clue to the mechanism by which complement produces membrane damage to these multicompartment liposomes because it suggests that antigen need not be present in all the lipid bilayers. Additional experiments are described that indicate the advantages to be gained by substitution of the dinitrophenylated derivatives for the naturally occurring amphipathic antigens (*e.g.*, ceramides and lipopolysaccharides) previously employed in the generation of liposomes subject to immune damage.

Since 1968, this laboratory has been utilizing liposomal model membranes to study the molecular basis of immune cytolysis. The properties of these liposomes, which release trapped glucose marker in the presence of an appropriate antiserum and complement source, have been recently reviewed in detail (Kinsky, 1972). The principle results of these investigations may be summarized as follows. Immunologically responsive liposomes can be prepared from defined mixtures containing four lipid constituents: a phospholipid (*e.g.*, lecithin and/or sphingomyelin), a sterol (*e.g.*, cholesterol), a charged amphiphile (*e.g.*, dicetyl phosphate or stearylamine), and an amphipathic antigen (see below). Of these constituents, only phospholipids are invariably present in natural membranes susceptible to immune cytolysis. Using liposomes prepared with radioactive phospholipids (Inoue and Kinsky, 1970) or synthetic nonhydrolyzable analogs of lecithin (Kinsky *et al.*, 1971), no evidence was obtained to support the contention that complement dependent membrane damage occurs by enzymatic degradation of phospholipids. As an alternative, these findings suggested that the cytolytic activity of complement may be due to disruption of the noncovalent bonds that are responsible for maintaining lipids in a stable bilayer configuration.

In these earlier investigations, sensitization of liposomes toward antibody–complement was accomplished with two different classes of naturally occurring amphipathic antigens. These were either various ceramide derivatives such as Forssman, globoside I, and galactocerebroside (Kinsky *et al.*, 1969; Inoue *et al.*, 1971), or different *S* and *R* form lipopolysaccharides isolated from mutants of *Salmonella minnesota* (Kataoka *et al.*, 1971a). Forssman-sensitized liposomes were the first to be examined because this compound is the main antigenic determinant responsible for the immune hemolysis of sheep erythrocytes which have long been the favored test object for studies on complement mechanism. In the context of the present investigation, it is important to emphasize that successful preparation of immunologically responsive liposomes with Forssman, or any of the other ceramide antigens, occurred only under conditions of "active sensitization." As defined previously (Kataoka *et al.*, 1971a), active sensitization means that the antigen was present at the time the lipids were dispersed to form the model membrane. These findings were the basis for the initial conclusion (Alving *et al.*, 1969) that the ceramide antigens were an integral part of the lipid bilayers that constitute the liposomes, *i.e.*, that the antigen had to be actually incorporated into the lipid bilayers in order to confer sensitivity toward antibody–complement. This conclusion was further supported by the fact that untreated *S* and *R* form lipolysaccharides, which are structurally quite different from the ceramide antigens, were also effective only under conditions of active sensitization (Kataoka *et al.*, 1971a).

In contrast, *S* and *R* form lipopolysaccharides that had undergone mild alkali treatment (1-hr incubation at 55–60° with 0.25–0.5 N NaOH) exhibited a significant anomalous property. Not only were the alkali treated lipopolysaccharides able to sensitize liposomes actively, but they were also effective under conditions of "passive sensitization" (Kataoka *et al.*, 1971a). In the passive sensitization procedure, the liposomes

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are formed initially in the absence of any antigen and then incubated with the latter, *i.e.*, after the model membrane had been generated. Subsequent experiments (Kataoka *et al.*, 1971b) demonstrated that lipid A, which was isolated from the lipopolysaccharides and then subjected to similar alkali treatment, could also passively sensitize liposomes to the action of specific anti-lipid A antibodies and complement. This finding indicated that the ability of treated lipopolysaccharides to passively sensitize liposomes could be attributed to the removal by alkaline hydrolysis of fatty acids esterified to the lipid A portion of these macromolecular antigens, rather than to some hitherto undetected alteration in the polysaccharide (O antigen or core) regions. Two other noteworthy characteristics of the alkali-treated materials were a greater solubility in water and, particularly in the case of lipid A, increased lytic activity towards erythrocytes (Galanos *et al.*, 1971).

The preceding observations implied a superficial resemblance between alkali treated lipopolysaccharides (or lipid A) and lysoglycerophospholipids; the latter compounds, almost by definition, manifest a much greater hemolytic potency than the parent phospholipids. This parallelism suggested, in turn, that appropriate lysoglycerophospholipids should be capable of passive sensitization of liposomes. To test this hypothesis, we have synthesized a homologous series of antigens: specifically, the dinitrophenylated derivatives of phosphatidylethanolamine, lysophosphatidylethanolamine, and glycerophosphorylethanolamine. The results presented in this paper demonstrate that only Dnp-lysoPE[1] can both actively and passively sensitize liposomes; Dnp-PE is effective solely under active sensitization conditions, whereas Dnp-GPE is incapable of sensitizing liposomes toward antibody–complement in either procedure. Additional experiments indicate several advantages associated with the use of Dnp-PE and Dnp-lysoPE as antigens for the preparation of liposomes suitable for the study of immune lytic mechanism.

Materials and Methods

Lipids. The sources for egg lecithin, beef brain sphingomyelin, cholesterol, dicetyl phosphate, and stearylamine were the same as those cited in a previous publication (Kinsky *et al.*, 1969). Egg lysophosphatidylethanolamine was purchased from the Pierce Chemical Co., Rockford, Ill. Phosphatidylethanolamine was isolated from *Azotobacter agilis* (Law and Essen, 1969); initial experiments were performed with a sample that was kindly provided by Dr. John Law, Department of Biochemistry, University of Chicago. Purity of all phospholipids was routinely confirmed by chromatography on commercial silica gel thin-layer plates (Brinkmann Instruments Inc., Westbury, N. Y.) using chloroform–methanol–water (70:30:5) as solvent system.

Cofactors and Enzymes for the Glucose Assay Reagent. ATP and TPN were obtained from Sigma Chemical Co., St. Louis, Mo.; yeast hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer-Mannheim Corp., New York, N. Y. Prior to use, the enzymes were dialyzed against cold distilled water to remove ammonium sulfate. Preparation of the "complete" and "incomplete" glucose assay reagents has been described previously (Kinsky *et al.*, 1969). The complete reagent contained 100 mM Tris-HCl (pH 7.5), 1 mM TPN, 2 mM ATP, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 64 mM NaCl, and approximately 80 μg of dialyzed hexokinase and 40 μg of dialyzed glucose-6-phosphate dehydrogenase per ml; the incomplete reagent had a similar composition except that TPN was omitted.

Immunologic Reagents. Rabbit antibodies against Dnp-hemocyanin were generously donated by Dr. H. N. Eisen and Mr. E. S. Simms (Department of Microbiology, Washington University School of Medicine) or obtained from Mr. Walter Gray (Gateway Immunosera Co., Cahokia, Ill.). The procedures used in their purification have been described in detail elsewhere (Eisen *et al.*, 1967). Prior to use, the antibodies were dialyzed against a solution of cold 150 mM NaCl–5 mM Tris (pH 7.4) to remove any phosphate ions introduced during preparation. Insoluble material, if any, was removed by centrifugation after dialysis and the antibody concentration in the supernatant solution was determined from the relative absorbancies at 278 and 360 mμ using the empirical formula given by Eisen *et al.* (1967). Purified normal rabbit γ-globulin (kindly supplied by Dr. Julian Fleischman, Department of Microbiology, Washington University School of Medicine) was treated in the same way. Guinea pig serum served as complement source in the present investigation. As in previous studies (see, *e.g.*, Kinsky *et al.*, 1969), the guinea pig serum was dialyzed against cold Veronal-buffered saline (prepared according to the method of Mayer, 1961) to reduce the level of endogenous glucose.

Synthesis and Purification of Dnp Derivatives. The procedures detailed below were developed to produce the desired products that moved as pure yellow compounds upon thin-layer chromatography in several solvent systems and gave the anticipated reactions toward ninhydrin and iodine vapor (Figure 1). We are indebted to Dr. Ralph A. Bradshaw (Department of Biochemistry, Washington University School of Medicine) for advice regarding the synthesis of the dinitrophenylated derivatives.

Dnp-PE. Phosphatidylethanolamine (122 mg equivalent to *ca.* 150 μmoles) was dissolved in 15 ml of chloroform containing 0.1 M triethanolamine. Approximately 750 μmoles of 2,4-dinitrofluorobenzene (Aldrich Chemical Co., Milwaukee, Wis.) dissolved in 375 μl of chloroform was then added with constant stirring. After 10 min at room temperature, the entire mixture was placed on a column prepared from 24 g of Unisil (Clarkson Chemical Co., Williamsport, Pa.) that had been previously washed with successive 250-ml portions of chloroform, methanol, and again chloroform. The column (dimensions 1.6 × 28 cm) was developed rapidly to minimize any possible hydrolysis of Dnp-PE that could occur during prolonged incubation under the alkaline reaction conditions. The first fraction, obtained by elution with 120 ml of chloroform, was discarded. The second fraction, obtained by elution with 360 ml of a 10:1 chloroform–methanol mixture, was dried under reduced pressure at 40°, taken up in 13 ml of chloroform, and rechromatogramed on another Unisil column (dimensions comparable to the above). After passage of 350 ml of chloroform, the fraction eluted with 750 ml of a 13:1 chloroform–methanol mixture was dried and dissolved in chloroform (15 ml). Overall yield of pure Dnp-PE in this fraction was 80% on the basis of total phosphate analysis.

Dnp-lysoPE. This derivative was synthesized essentially by the same procedure described above for Dnp-PE except that the reaction was performed on a smaller scale and only a single column was used for purification. Lysophosphatidylethanolamine (21 mg equivalent to *ca.* 40 μmoles) was dis-

[1] Abbreviations used are: Dnp-PE, dinitrophenylated phosphatidylethanolamine (diacylglycerophosphoethanolamine); Dnp-lysoPE, dinitrophenylated lysophosphatidylethanolamine (monoacylglycerophosphoethanolamine); Dnp-GPE, dinitrophenylated glycerophosphorylethanolamine.

solved in 4 ml of chloroform containing 0.1 M triethanolamine. After addition of dinitrofluorobenzene (*ca.* 200 μmoles dissolved in 100 μl of chloroform) and incubation for 10 min at room temperature, the reaction mixture was placed on a washed Unisil column (20 g; *ca.* 1.6 × 24 cm). The column was eluted successively with 180 ml of chloroform and 400 ml of a 13:1 chloroform–methanol mixture; both fractions were discarded. The next fraction, obtained by elution with 350 ml of a 7:1 chloroform–methanol mixture, was taken to dryness and redissolved in 4 ml of 1:1 chloroform–methanol; this fraction contained 29 μmoles of pure Dnp-lysoPE (73% yield).

Dnp-GPE. Synthesis of this derivative was accomplished by mild alkaline hydrolysis of Dnp-lysoPE using a minor modification of the method developed by Dawson (1960) for phospholipid analysis. Dnp-lysoPE (20 μmoles) was dissolved in 8.3 ml of ethanol. After addition of 0.9 ml of 0.3 N NaOH and incubation for 20 min at 37° with intermittent shaking, the reaction mixture was passed through a column of Amberlite IRC-50 (3.5 g; *ca.* 1.6 × 10 cm). The resin previously had been converted to the H^+ form with 10 ml of 1 N HCl followed by washes of 1 l. of water and 50 ml of cold 80% ethanol. The initial eluate and the fraction collected with 20 ml of cold 80% ethanol were combined and taken to dryness. The residue was suspended in 2.4 ml of water and mixed thoroughly with 2 ml of a 2:1 chloroform–isobutyl alcohol mixture, and the aqueous phase recovered by centrifugation. Interfacial material and the organic phase were reextracted four more times with 1 ml of water. The combined aqueous phases were then extracted with 3 ml of *n*-hexane prior to lyophilization. Phosphate analysis on this material, which was completely water soluble, indicated a 87% yield of pure Dnp-GPE.

Glycerophosphorylethanolamine (for use as a chromatographic standard; see Figure 1) was prepared by the above method starting with phosphatidylethanolamine.

Liposome Preparation. Liposomes were generated by the same procedure employed in all of our preceding investigations (see, *e.g.*, Kinsky *et al.* (1969) and Katoaka *et al.* (1971a) for complete details). In brief, this involved deposition of a "basic" lipid film (composition: 2 μmoles of lecithin or sphingomyelin, 1.5 μmoles of cholesterol, and 0.2 μmole of dicetyl phosphate) onto the walls of a 10-ml conical flask. The dried lipid film was then dispersed, using a Vortex mixer and a small quantity of glass beads in 0.2 ml of 300 mM glucose to yield a 10 mM phospholipid suspension. The liposome preparation was subsequently dialyzed for 1.5 hr at room temperature against 125 ml of an isotonic salt solution (75 mM NaCl–75 mM KCl) to remove most of the glucose marker that had not been trapped. This standard procedure was modified in several ways depending on the requirements of the particular experiment to produce actively and passively sensitized liposomes; the essential features of these modifications are described below (legends to tables and figures should be consulted for exact details).

In the case of active sensitization of liposomes with either Dnp-PE or Dnp-lysoPE, these compounds (dissolved in either chloroform or a 1:1 chloroform–methanol mixture) were added to the solution of the "basic" lipids prior to removal of the organic solvents under reduced pressure. This method could not be employed with Dnp-GPE because the latter was insoluble in chloroform and/or methanol. In this case, the dried lipid film was dispersed in an aqueous solution containing 300 mM glucose and the appropriate amount of Dnp-GPE. For comparative purposes, it has proven convenient to

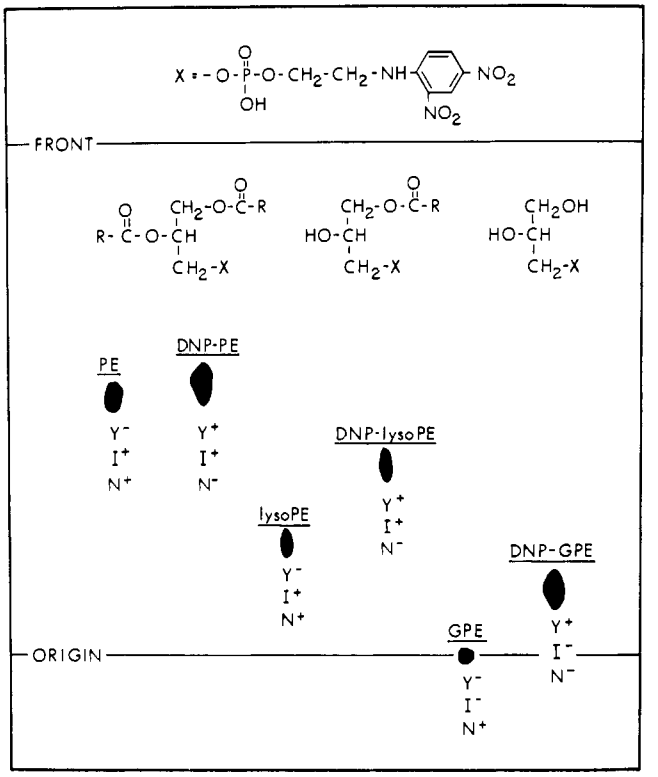

FIGURE 1: Structure and chromatographic behavior of dinitrophenylated phosphatidylethanolamine derivatives. Approximately 25–50 μg of each derivative was spotted on a silica gel thin-layer plate (0.25 mm thickness) which was developed in a solvent system of chloroform–methanol–water (70:30:5, v/v). Comparable quantities of phosphatidylethanolamine, lysophosphatidylethanolamine, and glycerophosphorylethanolamine were also run. Compounds were visualized by their yellow color and the plate was exposed to iodine vapor; after removal of the iodine by sublimation *in vacuo*, the plate was sprayed with a ninhydrin solution. + or − denotes, respectively, the presence or absence of yellow color (Y) and a positive or negative reaction towards iodine (I) and ninhydrin (N).

characterize such actively sensitized liposomes on the basis of the per cent antigen (relative to phospholipid) that was present at the time the model membrane was generated; thus, a liposome preparation actively sensitized with 10% antigen means that 200 nmoles of a dinitrophenylated derivative was added for every 2 μmoles of lecithin or sphingomyelin in the basic lipid mixture.

In the passive sensitization experiments, the dialyzed liposomes (prepared from the "basic" lipids only) were incubated with an aqueous solution of Dnp-GPE, or suspensions of Dnp-PE or Dnp-lysoPE. To prepare the latter, a suitable volume of the stock solution was added to a small test tube and taken to dryness under a stream of N_2; the tubes were then placed in a desiccator under continuous evacuation (0.05 mm) for at least 1 hr to ensure complete removal of the organic solvents. The dried compounds were dispersed with a Vortex mixer in an isotonic buffer to give suspensions of the desired concentrations (usually ranging from 20 μM to 1 mM). Sonication was not required because, under the conditions employed, the suspensions of Dnp-PE or Dnp-lysoPE were homogeneous and displayed only slight opalescence.

Glucose Release Assay. The amounts of total, untrapped, and (by difference) trapped glucose in the liposome preparations were measured by the methods that have been described in detail elsewhere (Kinsky *et al.*, 1969, 1970). As in all of our

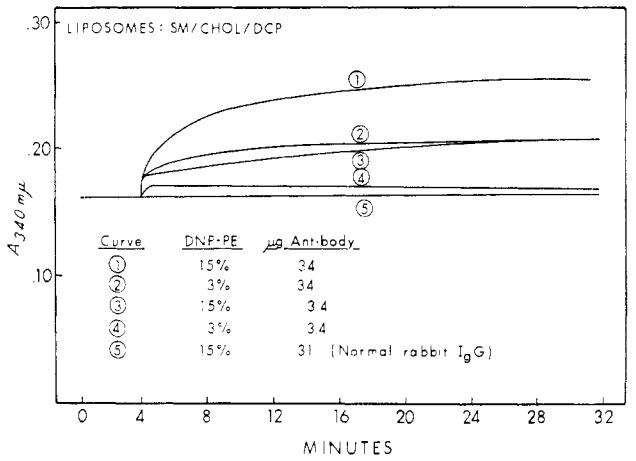

FIGURE 2: Light-scattering changes due to liposome agglutination by antibody. Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing either 3 or 15% antigen (Dnp-PE). Five microliters of each liposome preparation was added to cuvets containing 500 μl of incomplete assay reagent and 495 μl of Veronal-buffered saline. After 4 min, the cuvets received the indicated amounts (in 5 μl) of antibody (preparation GS-1) or normal rabbit γ-globulin (IgG). Absorbancy was monitored continuously in a Gilford recording spectrophotometer; slight differences in the initial absorbance values (before antibody) were offset to provide a common base line.

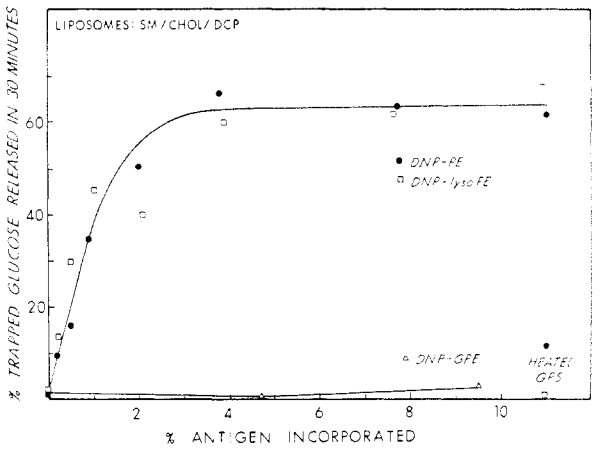

FIGURE 3: Active sensitization of liposomes by Dnp-PE, Dnp-lyso-PE, and Dnp-GPE. Liposomes were prepared from sphingomyelin cholesterol–dicetyl phosphate mixtures containing varying amounts of each of the Dnp derivatives to give the percent antigen content indicated on the abscissa. Assays were performed by the standard procedure in the presence of 113 μl of guinea pig serum (GPS) and 36 μg of antibody (preparation GS-1). In the control experiments, liposomes sensitized with excess Dnp-PE or Dnp-lysoPE were assayed under identical conditions except that the guinea pig serum had been previously incubated at 56° for 30 min before addition to the cuvets.

previous studies, results are expressed as the percentage of trapped glucose released under a given set of experimental conditions. However, for reasons discussed below, we have found it necessary to modify slightly the spectrophotometric procedure that has been used to determine marker loss in the presence of antibody and complement.

The original standard assay employed in earlier investigations (see, *e.g.*, Kinsky *et al.*, 1969, and Kataoka *et al.*, 1971b) was performed as follows. For each liposome preparation to be examined a "complete" cuvet was set up containing 500 μl of complete assay reagent, the desired amounts of antibody (ranging from 0 to 36 μg) and dialyzed guinea pig serum (ranging from 0 to 113 μl), and sufficient Veronal-buffered saline to give a volume of 995 μl. The absorbancy at 340 mμ was recorded and then the reaction initiated by the addition of 5 μl of liposomes; the absorbancy was again determined after 30-min incubation at room temperature (*ca.* 22°). Subtraction of the initial value from the final value provided a measure of the amount of glucose released *plus* the contributions to the absorbancy of any untrapped glucose in, and light scatter by, the liposome preparation. To correct for the latter variables, the above procedure was repeated in a "complete" assay cuvet that contained Veronal-buffered saline instead of antibody and guinea pig serum. The corrected difference could be used to calculate the percentage of trapped marker released—provided that light scatter by the liposomes did not change during the course of the reaction; this condition was satisfied in the preceding investigations on liposomes sensitized with either ceramide antigens or lipopolysaccharides.

In contrast, preliminary experiments indicated that antibody alone can cause appreciable light-scattering changes in liposomes sensitized with various Dnp derivatives (particularly Dnp-PE); this was revealed by an increase in the absorbancy at 340 mμ in cuvets containing "incomplete" assay reagent. Figure 2 (curve 1) shows that the increase was especially pronounced when liposomes prepared with a high amount of antigen (15%) were incubated with a high amount of antibody (34 μg/ml). Smaller light-scattering changes were observed when the antigen content of the liposomes and/or the concentration of antibody were reduced (curves 2, 3, and 4). No change occurred upon incubation of liposomes with high antigen content in the presence of a high concentration of normal rabbit γ-globulin (curve 5) indicating that the increased absorbancy was due to antibody-induced agglutination of liposomes. Accordingly, before calculation of the percentage glucose released in the presence of antibody and complement, the corrected difference referred to in the preceding paragraph had to be further corrected by any changes in liposomal light scatter which developed during the course of the reaction. This was simply accomplished by running in parallel an "incomplete" assay identical with the "complete" assay described above except that the cuvets contained incomplete reagent.

Miscellaneous. Phospholipid concentration was determined by minor modification of the method used by Gerlach and Deuticke (1963) for total phosphate analysis. Antibody binding to liposomes was measured by the experimental procedure described in the legend to Figure 5.

Results

Active Sensitization by Dnp-PE. Dnp-PE can actively sensitize liposomes prepared with either sphingomyelin or lecithin as phospholipid. This is illustrated in Figure 3 for sphingomyelin liposomes; an essentially similar saturation curve was obtained for actively sensitized lecithin liposomes (not shown). In the case of sphingomyelin liposomes, saturation was achieved with 4% Dnp-PE, *i.e.*, 1 molecule of antigen for every 25 molecules of phospholipid. This ratio is appreciably greater than that previously derived for the active sensitization of analogous liposomes by Forssman antigen where near maximum sensitization was obtained with 1 molecule of the ceramide for approximately every 250 molecules

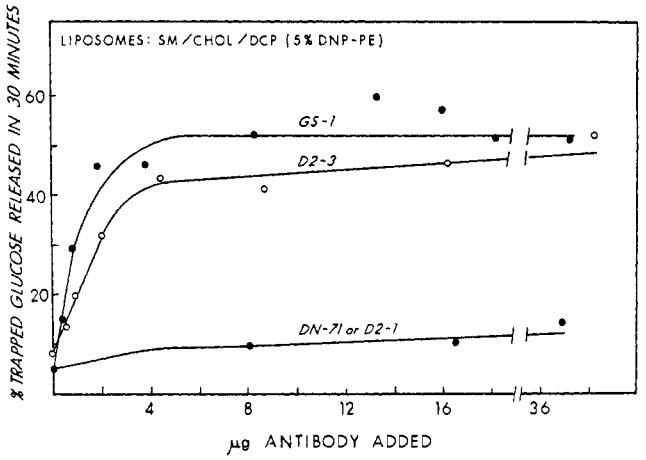

FIGURE 4: Effect of antibody concentration on glucose release from actively sensitized liposomes. Liposomes were prepared from a sphingomyelin–cholesterol–dicetyl phosphate mixture containing 5% Dnp-PE. Assays were performed by the standard procedure in the presence of 113 μl of guinea pig serum and varying amounts of antibody as indicated on the abscissa. GS-1, D2-3, DN-71, and D2-1 refer to the lot numbers of different antibody preparations whose properties are further described in the text.

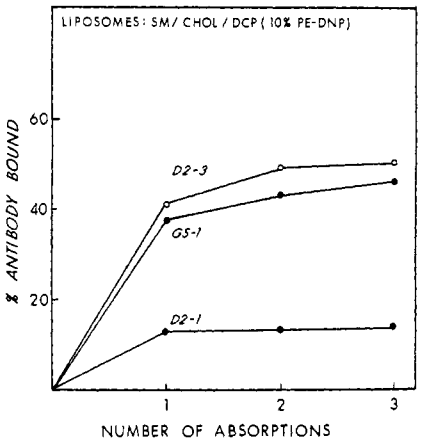

FIGURE 5: Binding of different antibody preparations to actively sensitized liposomes. Liposomes were prepared from a sphingomyelin–cholesterol–dicetyl phosphate mixture containing 10% Dnp-PE; they were swollen in 150 mM NaCl instead of isotonic glucose solution. Adsorption was performed as follows. Tubes contained initially 500 μg of the indicated antibody preparations dissolved in 1 ml of 150 mM NaCl–10 mM phosphate (pH 7.5); absorbance at 278 and 360 mμ was recorded and then 98 μl of liposomes was added. After 30 min at room temperature, the incubation mixtures were centrifuged for 30 min at 35,000 rpm (Spinco No. 40 rotor) and the absorbance of the supernatant solutions was again determined. This procedure was repeated two more times by the addition of 98 μl of liposomes to the supernatant solutions resulting from the previous adsorption. All absorbance values were corrected for dilution before calculation of the amount of antibody recovered by the formula of Eisen *et al.* (1967).

of sphingomyelin (Kinsky *et al.*, 1969). It should, however, be noted that the anti-Dnp antibodies used in the present investigation are exclsuviely of the IgG class (H. N. Eisen, E. R. Simms, and W. Gray, personal communication), whereas at least 90% of the Forssman antibodies in the commercial rabbit antisheep erythrocyte employed in our earlier studies belong to the IgM class (unpublished observations). For a number of years (see, *e.g.*, Borsos and Rapp, 1965), it has been generally accepted that a single membrane bound antigen–antibody complex is able to activate the complement sequence when formed with IgM antibodies, whereas a minimum of two antigen–antibody complexes in close proximity is required when the immune complex involves IgG antibodies. The preceding quantitative considerations are entirely consistent with this conclusion.

Effect of Antibody Affinity. In the course of this study, several different purified anti-Dnp antibody preparations were examined for their ability to release glucose in the presence of complement from liposomes that had been actively sensitized with Dnp-PE. Figure 4 shows that not all antibody preparations were equally active in this regard; lot GS-1 and D2-3 caused significant loss of marker in contrast to lots DN-71 and D2-1 which had only a slight effect. Subsequent experiments (Figure 5) revealed that approximately 50% of the protein present in antibody preparations GS-1 and D2-3 could be bound to sphingomyelin liposomes actively sensitized with excess Dnp-PE. Under identical conditions, only slightly more than 10% of the protein in preparation D2-1 was absorbed (experiments with lot DN-71 were not performed due to scarcity of material).

In connection with these observations, it should be pointed out that preparations GS-1 and D2-3 were both derived from rabbits under long-term immunization conditions that lead to the production of high-affinity antibodies (Eisen *et al.*, 1967). "Affinity" here refers to the average intrinsic association constant (K_0) of the antibodies for the hapten, ε-Dnp-lysine, which is the predominant antigenic determinant in the immunogen, Dnp-hemocyanin. The presence of high-affinity antibodies in these lots was kindly confirmed for us by Mr. Walter Gray; fluorescence quenching measurements (Eisen and McGuigan, 1971) indicated a K_0 of approximately 10^8 l. mole^{-1}. In contrast, preparations DN-71 and D2-1 had an average intrinsic association constant of approximately 10^5 l. mole^{-1}. It is of course recognized that the principle moiety in the liposomes responsible for antibody binding is Dnp-phosphorylethanolamine which differs considerably from ε-Dnp-lysine in that two methylene groups are replaced by a negatively charged phosphoryl residue (Figure 1). The above findings are nevertheless consistent with the prevailing view (*cf.* discussion in Eisen, 1967) that cross-reacting ligands are bound more strongly by antibodies with a greater affinity for the homologous hapten, *i.e.*, higher affinity antibodies manifest a broader specificity. In preliminary experiments, this was substantiated by the observation that Dnp-GPE was able to quench the fluorescence of the high-affinity antibody preparations but had little effect on the one low-affinity preparation tested. Dnp-GPE did not affect the fluorescence after the high-affinity preparations had been absorbed with liposomes actively sensitized with Dnp-PE although the supernatant solutions were still moderately quenchable by ε-Dnp-lysine. This finding suggests that, even in the high-affinity preparations, not all of the antibodies are capable of reacting with Dnp-phosphorylethanolamine, in agreement with the direct binding experiments illustrated in Figure 5.

Active Sensitization by Dnp-lysoPE; Hapten Inhibition by Dnp-GPE. Figure 3 shows that Dnp-lysoPE was equally as effective as Dnp-PE in the active sensitization of sphingomyelin liposomes. However, Dnp-GPE was unable to actively sensitize liposomes over the concentration range examined. Liposomes, particularly those prepared in the presence of high levels of Dnp-GPE, were nevertheless visibly yellow; this was presumably due to trapping of the derivative in the aqueous compartments of the liposomes rather than its incor-

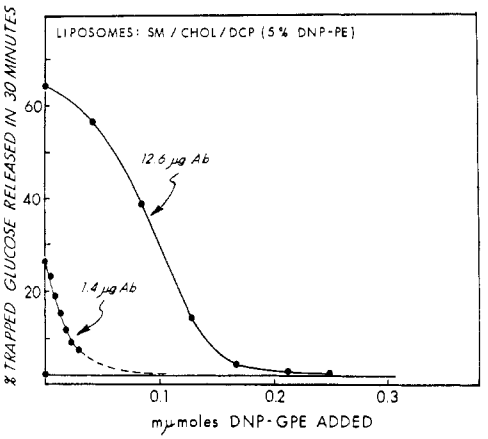

FIGURE 6: Inhibition by Dnp-GPE of glucose release from actively sensitized liposomes. Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing 5% Dnp-PE. Assays were performed by the standard procedure in the presence of 113 μl of guinea pig serum, 1.4 or 12.6 μg of antibody (preparation GS-1), and varying amounts of Dnp-GPE as indicated on the abscissa. The base line was established by incubation of liposomes in assay cuvets containing only guinea pig serum (no antibody or Dnp-GPE).

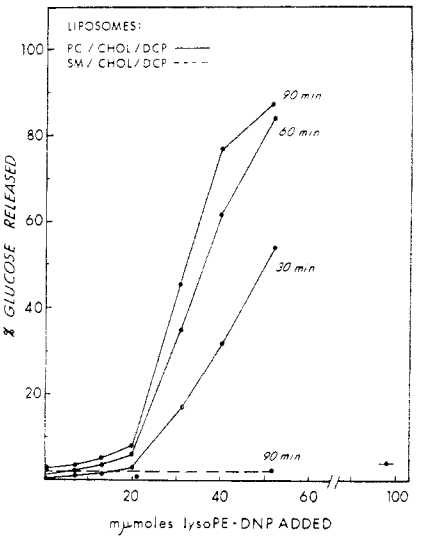

FIGURE 7: Effect of Dnp-lysoPE on liposomes. Liposomes were prepared from mixtures of lecithin (or sphingomyelin)–cholesterol–dicetyl phosphate. Assays were performed essentially by the same procedure used to determine glucose release in the presence of antibody and complement. Cuvets contained initially 500 μl of complete or incomplete assay reagent, varying amounts of Dnp-lyosPE as indicated on the abscissa, and sufficient Veronal-buffered saline to give a volume of 995 μl. After measurement of the absorbance at 340 mμ, the reaction was started by the addition of 5 μl of liposomes; absorbance was again determined after 30, 60, and 90-min incubation at room temperature.

poration into the lipid bilayers (see Discussion). Failure to obtain glucose release from these liposomes could not be attributed to a lower affinity of Dnp-GPE for the antibodies compared to either of the other dinitrophenylated derivatives. As illustrated in Figure 6, Dnp-GPE could function as a water soluble hapten whose addition completely inhibited marker loss from liposomes actively sensitized with Dnp-PE.

Additional Properties of Actively Sensitized Liposomes. Figures 3 and 4 demonstrate, respectively, that the extent of glucose release is dependent on the amount of antigen incorporated into the liposomes and the concentration of antibody in the assay cuvets. Also, it should be noted that antibody alone did not promote marker loss but required the presence of a native complement source; heating of the guinea pig serum for 30 min at 56° (conditions usually employed to inactivate hemolytic complement activity) abolished glucose release (Figure 3). These observations are by no means novel; previous studies (reviewed in Kinsky, 1972) have shown that liposomes actively sensitized with Forssman, globoside I, galactocerebroside, lipopolysaccharides, and lipid A manifest a similar behavior. Extensive experiments were nevertheless performed to determine if there was any essential difference between liposomes actively sensitized with Dnp-PE (or Dnp-lysoPE) and the other amphipathic antigens. None was found and therefore it seems sufficient to record briefly the following observations simply to emphasize those properties which actively sensitized liposomes share in common.

Titrations (similar to those described in Figure 4) using liposomes containing varying levels of Dnp-PE revealed an inverse correlation between the concentration of antibody required to produce a given percentage of glucose release and the amount of antigen incorporated (*cf.* Kinsky *et al.*, 1969). Similarly, more native guinea pig serum was necessary to affect a given percentage of marker loss when less of the antigen was incorporated (*cf.* Kinsky *et al.*, 1969). In the presence of excess antibody and complement source, liposomes prepared with lecithin released more glucose than liposomes prepared with sphingomyelin; nonimmune damage (*i.e.*, marker release occurring in the absence of antibody and/or guinea pig serum) was also greater from lecithin liposomes than from sphingomyelin liposomes (*cf.* Inoue *et al.*, 1970; Kataoka *et al.*, 1971a,b). Positively charged liposomes (obtained by substitution of stearylamine for dicetyl phosphate in the basic lipid mixture) released nearly as much glucose as negatively charged liposomes (*cf.* Kinsky *et al.*, 1969). Finally, kinetic experiments demonstrated that marker was released at a faster rate and with a shorter lag phase from liposomes that contained more Dnp-PE (*cf.* Kinsky *et al.*, 1969).

Effect of Dnp Derivatives on Lecithin and Sphingomyelin Liposomes. Prior to the passive sensitization experiments, it was necessary to determine if incubation of liposomes with the dinitrophenylated derivatives alone could cause loss of marker under the conditions of assay. No effect on lecithin and sphingomyelin liposomes was obtained with either Dnp-PE or Dnp-GPE at final concentrations approaching 10^{-4} M; higher concentrations could not be feasibly tested because of the appreciable absorption at 340 mμ by the Dnp chromophore. Figure 7 shows, however, that Dnp-lysoPE could produce extensive glucose release from lecithin liposomes in a time-dependent reaction above a critical threshold concentration of 20 nmoles/ml (2×10^{-5} M). In contrast, Dnp-lysoPE could not induce loss of marker from sphingomyelin liposomes under similar conditions (Figure 7). The latter observation was considered significant because it provides another indication that sphingomyelin–cholesterol bilayers may be intrinsically more stable than lecithin–cholesterol bilayers (see discussion in Kinsky (1972) for earlier evidence).

Passive Sensitization by Dnp-lysoPE. The experiments summarized in Table I demonstrate that Dnp-lysoPE can passively sensitize liposomes prepared with either lecithin or sphingomyelin. The following observations indicate that glucose release from these liposomes reflects a genuine immune response. Normal rabbit γ-globulin (IgG) cannot substitute

TABLE I: Requirements for Glucose Release from Passively Sensitized Liposomes.[a]

Component Omitted or Added	% Glucose Released in 30 min from Liposomes Prepared with: Lecithin	Sphingomyelin
None	68.2	38.2
−Ab, +normal IgG	9.1	1.5
−Native GPS, +heated GPS	8.0	0.6
−Dnp-lysoPE, +lysoPE	8.7	0.9

[a] Liposomes were prepared from mixtures of lecithin (or sphingomyelin)–cholesterol–dicetyl phosphate. Passive sensitization was performed in cuvets containing 500 μl of complete assay reagent, 5 μl of liposomes, 1 nmole of Dnp-lysoPE (or lysoPE), and sufficient Veronal-buffered saline to give a volume of 875 μl. After 30-min preincubation at room temperature, 113 μl of native (or heated) guinea pig serum (GPS) was added and the absorbancy at 340 mμ was determined. The reaction was initiated by the addition of 36 μg (12 μl) of GS-1 antibody (or normal rabbit γ-globulin) and the absorbancy again measured 30 min later. Per cent glucose released was calculated from the difference between the final and initial values after correction for any changes occurring in identical cuvets that contained incomplete, instead of complete, reagent.

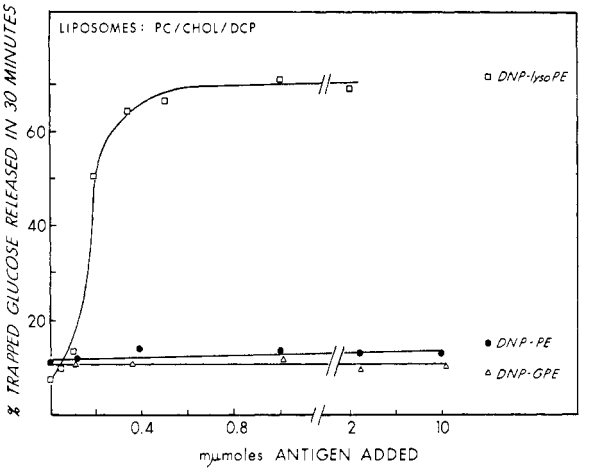

FIGURE 8: Passive sensitization of lecithin liposomes by Dnp-PE, Dnp-lysoPE, and Dnp-GPE. Liposomes were prepared from a mixture of lecithin–cholesterol–dicetyl phosphate. Passive sensitization was performed in cuvets containing 500 μl of complete assay reagent, varying amounts of each of the Dnp derivatives as indicated on the abscissa, and sufficient Veronal-buffered saline to give a volume of 875 μl. Cuvets were preincubated 30 min at room temperature before the addition of 113 μl of guinea pig serum. Absorbancy at 340 mμ was measured and then the reaction started by the addition of 12 μl (36 μg) of GS-1 antibody. Absorbancy was again determined 30 min later. The difference between the final and initial values was used to calculate the percent glucose release after correction for any changes that occurred in identical cuvets containing incomplete, instead of complete, assay reagent.

for the antibody; decomplemented (heated) guinea pig serum cannot replace native guinea pig serum; lysophosphatidylethanolamine (which lacks the Dnp antigenic determinant) is ineffective.

Antigen titration curves for passive sensitization of lecithin and sphingomyelin liposomes are presented in Figures 8 and 9. It is important to emphasize that the concentration of Dnp-lysoPE required for maximum sensitization of lecithin liposomes is significantly less than the concentration which *per se* causes glucose release; this obviously applies to sphingomyelin liposomes where Dnp-lysoPE was found to have no effect over the range tested (*cf.* Figure 7). It should also be noted that passively sensitized sphingomyelin liposomes release less marker than lecithin liposomes in the presence of antibody–complement (compare Figures 8 and 9), in line with the greater stability of sphingomyelin liposomes toward a variety of lytic agents (Kinsky, 1972).

The kinetics of glucose release from passively sensitized lecithin liposomes is illustrated in Figure 10. The fastest rate of glucose release occurred when the complement source was added last; under these conditions (preincubation of liposomes with Dnp-lysoPE and antibody), loss of marker was not preceded by a lag phase. A short lag phase, as well as a slightly slower rate of marker release, was observed when the reaction was initiated by the addition of antibody. Considerably slower rates and longer lag phases were apparent when either Dnp-lysoPE or liposomes was the terminal component added. These findings are consistent with the view that glucose release from passively sensitized liposomes occurs in sequential stages. The first involves interaction of Dnp-lysoPE with the model membrane; this is followed by formation of antigen–antibody complexes on the liposomal surface which, in turn, trigger the complement reactions leading eventually to membrane damage.

Effect of Dnp-PE and Dnp-GPE. Dnp-PE could not passively sensitize liposomes prepared with either lecithin (Figure 8) or sphingomyelin (Figure 9). This is in marked contrast to its behavior under conditions of active sensitization where, as noted above, Dnp-PE was just as effective as Dnp-lysoPE (Figure 3). Passive sensitization of lecithin and sphingomyelin liposomes by Dnp-GPE was not obtained (Figures 8 and 9).

Column Chromatography of Passively Sensitized Liposomes. In preliminary experiments concerned with passive sensitization by Dnp-lysoPE, it was found that incubation of liposomes with concentrations above approximately 2 nmoles/ml

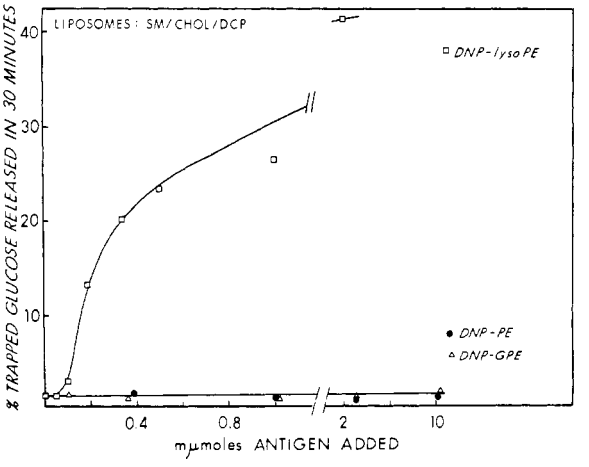

FIGURE 9: Passive sensitization of sphingomyelin liposomes by Dnp-PE, Dnp-lysoPE, and Dnp-GPE. Procedure identical with that described in the legend to Figure 8 except that the liposomes were prepared from a mixture of sphingomyelin–cholesterol–dicetyl phosphate.

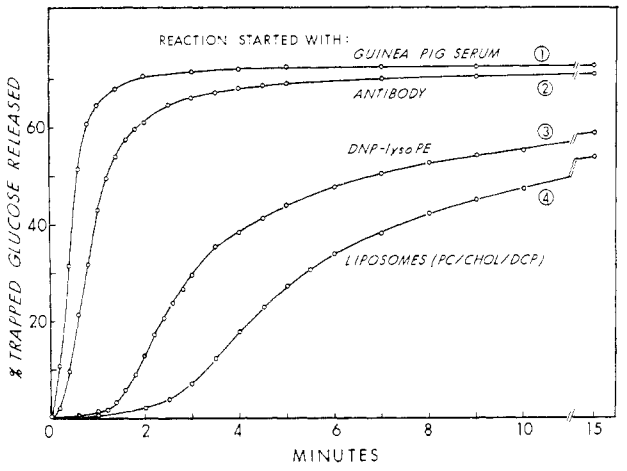

FIGURE 10: Effect of addition order on the kinetics of glucose release from passively sensitized liposomes. Liposomes were prepared from a mixture of lecithin–cholesterol–dicetyl phosphate. Passive sensitization was performed by a modification of the procedures described in the legends to Table II and Figure 8. All cuvets contained initially 318 μl of Veronal-buffered saline and 500 μl of the appropriate assay reagent (complete or incomplete); subsequent additions and preincubations were carried out in the following order. Curve 1: 51 μl of Dnp-lysoPE (1 nmole); 5 μl of liposomes; preincubation for 30 min; 12 μl of antibody (36 μg of GS-1); preincubation for another 5 min; reaction started by the addition of 113 μl of guinea pig serum. Curve 2: Dnp-lysoPE (51 μl), liposomes (5 μl); preincubation for 30 min; guinea pig serum (113 μl); preincubation for another 5 min; reaction started by the addition of antibody (12 μl). Curve 3: guinea pig serum (113 μl); antibody (12 μl); liposomes (5 μl); preincubation for 5 min; reaction started by the addition of Dnp-lysoPE (51 μl). Curve 4: Dnp-lysoPE (51 μl); guinea pig serum (113 μl); antibody (12 μl); preincubation for 5 min; reaction started by the addition of liposomes (5 μl). In all cases, absorbancy was monitored continuously following addition of the terminal reactant. Per cent glucose released was calculated as described previously (Table II and Figure 8) after correction for any absorbancy at 340 mμ due to terminal reactant *per se*.

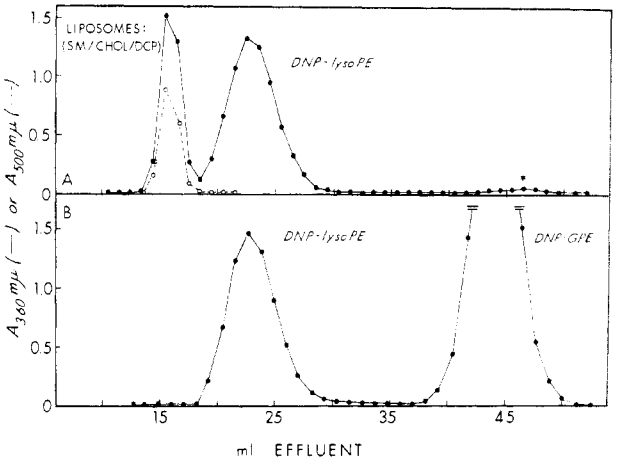

FIGURE 11: Chromatography of passively sensitized liposomes, Dnp-lysoPE, and Dnp-GPE. Liposomes were passively sensitized as indicated in the legend to Table II which should also be consulted for details of the chromatographic procedure. Panel A shows the elution pattern obtained after chromatography of 400 μl of the incubation mixture containing 1 μmole of Dnp-lysoPE; the presence of Dnp-lysoPE was determined by the absorbance of the effluent at 360 mμ. The appearance of liposomes was detected by the increased light scatter at either 360 or 500 mμ (at this wavelength, the Dnp chromophore manifests negligible absorbance). Panel B shows the elution pattern obtained after 1 μmole of Dnp-lysoPE and 1 μmole of Dnp-GPE were chromatogrammed individually on the same column used to separate passively sensitized liposomes from free Dnp-lysoPE.

TABLE II: Glucose Release from Passively Sensitized Liposomes before and after Chromatography on Sephadex G-200.[a]

Liposome Treatment	% Glucose Released in 30 min in the Presence of:	
	Native GPS	Heated GPS
Before G-200	7.3	4.9
After G-200	52.8	6.1

[a] Liposomes were prepared from a sphingomyelin–cholesterol–dicetyl phosphate mixture. 250 μl of liposomes was incubated with 250 μl of 5 mM Dnp-lysoPE dissolved in 10 mM Tris–150 mM NaCl (pH 7.5). After 30 min at room temperature, 11.5 μl of the incubation mixture was assayed for glucose release by the standard procedure in the presence of 36 μg of antibody (GS-1) and 113 μl of native or heated guinea pig serum (GPS). Another portion of the incubation mixture (400 μl) was chromatogrammed on a 1 × 55 cm Sephadex G-200 column previously equilibrated with the Tris–saline buffer; the latter was also employed for elution. After chromatography, 68 μl of the peak fraction that contained liposomes (eluted after passage of 16 ml of buffer; see panel A of Figure 11) was assayed for glucose release under the identical conditions described above.

inhibited glucose release. This was presumably due to the presence of free Dnp-lysoPE that had not been incorporated into the model membrane and could therefore function as a haptenic inhibitor; a similar phenomenon has been described above for Dnp-GPE (Figure 6). To test this hypothesis, passively sensitized sphingomyelin liposomes were subjected to chromatography on Sephadex G-200. The experiments reported in Table II show that liposomes, which did not respond to antibody–complement before chromatography, did so after separation from free antigen; the elution profile illustrated in Figure 11 (panel A) indicates that unincorporated Dnp-lysoPE was removed from these liposomes.

In a parallel experiment, equivalent amounts of Dnp-lysoPE and Dnp-GPE, which had not been incubated with liposomes, were passed through the same column to obtain some information about the physical properties of the antigen and the quantity bound to liposomes under conditions of passive sensitization. The elution pattern shown in Figure 11 (panel B) indicates that Dnp-lysoPE comes off a Sephadex G-200 column much earlier than Dnp-GPE although the molecular weight difference between these two derivatives is only that of a single fatty acid residue. This observation suggests that most of Dnp-lysoPE is present in micellar form under the experimental conditions employed; this conclusion is further supported by the fact that the total integrated absorbance of the Dnp-lysoPE peak is less than that of the Dnp-GPE peak. Comparison of the peak for unincorporated Dnp-lysoPE (panel A) to the corresponding peak in the control (panel B) reveals little difference in their areas, suggesting that only a small amount of the antigen is bound to passively sensitized liposomes. In this connection, it should be noted that we have consistently detected a very minute peak (indicated by an asterisk in panel A) after Sephadex G-200 chromatography of liposome–antigen incubation mixtures or Dnp-lysoPE incubated in the absence of liposomes. The position of

this peak suggests that it may correspond to the species of Dnp-lysoPE with which liposomes interact, *i.e.*, individual molecules of the antigen that are in true solution and in equilibrium with the micelles.

Discussion

The present investigation clearly indicates that antigenic compounds, which can be characterized as "hydrophobic," are capable of sensitizing liposomes to the action of antibody–complement. These results thus lend support to the hypothesis that the antigens are present in bilayer configuration along with the other lipid constituents (*i.e.*, phospholipid, sterol, and charged amphiphile). This conclusion is based on the observation that Dnp-PE and Dnp-lysoPE (compounds which possess at least one fatty acid residue) are effective in actively sensitizing liposomes whereas Dnp-GPE (which lacks any appreciable amphipathic character) is not.

Of even greater significance, in view of the previous studies on alkali-treated lipopolysaccharides and lipid A mentioned earlier, is the finding that Dnp-lysoPE (but not Dnp-PE) is capable of passively sensitizing liposomes. One of the principle goals of this investigation was to determine whether antigens, which are effective under conditions of passive sensitization, may be characterized by a higher critical micelle concentration favoring formation of an antigen species (perhaps monomeric) that can be more readily incorporated into liposomal bilayers. This now appears to be a valid assumption because intact glycerophospholipids generally possess much lower critical micelle concentrations (range 10^{-6}–10^{-9} M) than the corresponding lyso derivatives (range 10^{-3}–10^{-5} M).

The experiments dealing with the phenomenon of passive sensitization have also provided an important clue as to the mechanism by which complement may produce membrane damage to these multicompartment liposomes. It is a recognized dilemma that maximally sensitized liposomes release significantly more glucose than the outermost compartment would be expected to contain (approximately 10%; *cf.* calculations in Kinsky (1972)). This implies that an appreciable portion of the released marker must come from the "deeper" aqueous compartments as a consequence of damage to internal bilayers. On the basis of studies with actively sensitized liposomes, we have entertained the possibility that this might occur in successive stages as follows. Immune damage to the "first" (external) bilayer permits the entrance of antibody molecules and the components of the complement system; the antibodies then combine with antigens in the next bilayer; the resulting immune complexes initiate the complement cascade which destroys this "second" bilayer resulting in the exposure of the "third" bilayer, etc. The present study has shown, however, that passively sensitized liposomes (which presumably contain the antigen only in the external lipid bilayer) release as much trapped glucose as do actively sensitized liposomes (in which all the bilayers contain antigen) (*e.g.*, compare Tables I and II to Figures 3 and 4). Accordingly, it appears very unlikely that damage to internal bilayers proceeds by the classical complement sequence involving prior formation of an antigen–antibody complex. A current working hypothesis is that activation of the complement component responsible for membrane damage (activation occurring *via* immune complexes on the outermost bilayer) results in the exposure of hydrophobic regions in this protein (or release of fragments containing such regions); a portion of the latter would then diffuse to the "second" bilayer causing its destruction, etc. Although alternative explanations exist, this hypothesis might also account for the fact that complete (*i.e.*, 100%) glucose release was never obtained in our experiments because hydrophobic regions have, by definition, a limited survival time in water.

Finally, mention should be made of the other main goal that prompted this investigation. Many membrane-associated phenomena in addition to complement mechanism can be investigated with lipid model membrane systems and, for such purposes, it is essential to have available both an abundant supply of amphipathic antigen of known structure and the corresponding antibody. Unfortunately, none of the systems previously employed completely fulfills these requirements. For example, the commercial availability of high titer anti-Forssman serum (*i.e.*, rabbit antisheep erythrocyte serum) is offset by the difficulty in obtaining the pure antigen in good yield because natural sources (*e.g.*, sheep erythrocytes and horse kidney) contain little of this compound. Conversely, in the case of the other ceramide antigens, adequate amounts of globoside I and galactocerebroside are relatively easy to isolate from human (or porcine) erythrocytes and beef brain myelin, respectively. However, our experience has been that immunization of rabbits with these materials produces variable and low titer antisera. Neither of these problems exists with the lipopolysaccharides (*i.e.*, both antigens and high titer antisera can be readily obtained) but, unfortunately, the structure of these complex macromolecular antigens has not yet been completely elucidated.

All of these shortcomings have been circumvented with the synthetic dinitrophenylated derivatives of phosphatidylethanolamine and lysophosphatidylethanolamine as illustrated by the present experiments. Potentially, this approach can be extended by the attachment of antigenic determinants other than Dnp (*e.g.*, carbohydrates or peptides) to the amino group of these phospholipids; such experiments are now in progress.

Acknowledgments

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Utilization of a Cyclopentane Analog of Glutamate (*cis*-1-Amino-1,3-dicarboxycyclopentane) by Glutamine Synthetase†

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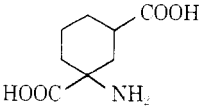
ABSTRACT: Previous studies, which showed that *cis*-1-amino-1,3-dicarboxycyclohexane is a substrate of ovine brain glutamine synthetase, have been extended to 1-amino-1,3-dicarboxycyclopentane, a glutamate analog in which two methylene groups have been introduced between the α- and γ-carbon atoms of glutamate. The cis and trans forms of 1-amino-1,3-dicarboxycyclopentane were separated and identified. Glutamine synthetase utilizes about 50% of the racemic cis form and does not interact with the trans form. Amide synthesis from *cis*-1-amino-1,3-dicarboxycyclopentane is more rapid than from *cis*-1-amino-1,3-dicarboxycyclohexane, and the K_m value for ammonia is much lower with the cyclopentane glutamate analog. These observations are in accord with computer-aided calculations of the conformation of the cyclopentane analog and its orientation on the active site of the enzyme. The findings are also consistent with earlier data on the mapping of the active site of glutamine synthetase which led to the conclusion that L-glutamate binds to the enzyme in the extended conformation.

The observation that *cis*-1-amino-1,3-dicarboxycyclohexane (A), a compound whose 5-carbon chain is much more restricted in movement than that of glutamate, is a good substrate of glutamine synthetase (Gass and Meister, 1970a) supports the hypothesis that L-glutamate binds to the active site of the enzyme in the fully (or almost fully) extended conformation with its α-hydrogen atom directed away from the enzyme (Meister, 1968; Gass and Meister, 1970b). The studies on 1-amino-1,3-dicarboxycyclohexane were initiated after it became apparent that glutamine synthetase can utilize α-methyl-L-glutamate, *threo*-β-methyl-D-glutamate, and *threo*-γ-methyl-L-glutamate, but not the other monomethyl-substituted glutamates (Kagan *et al.*, 1965; Kagan and Meister, 1966a,b); thus, 1-amino-1,3-dicarboxycyclohexane can be considered as a derivative of glutamate with a chain of three methylene groups introduced between the α- and γ-carbon atoms (Gass and Meister, 1970a).

In the present work we have prepared and studied an analogous glutamate derivative in which two methylene groups have been introduced between the α- and γ-carbon atoms of glutamate. The 5-carbon chain of this compound, 1-amino-1,3-dicarboxycyclopentane (B), is also very much more restricted in movement than that of glutamate. The findings reported here demonstrate that *cis*-1-amino-1,3-dicarboxycyclopentane is a good substrate of ovine brain glutamine synthetase and are in accord with earlier conclusions about the mapping of the active site of this enzyme (Meister, 1968; Gass and Meister, 1970b). The present studies suggest that this cyclopentane analog of glutamate may also be of interest in connection with other enzymes that act on glutamate.



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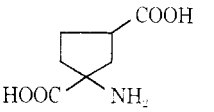

B

Experimental Section

Materials

Glutamine synthetase was isolated from sheep brain as described (Rowe *et al.*, 1970).

We are indebted to Dr. Leslie Hellerman for giving us a sample of 1-amino-1,3-dicarboxycyclopentane (mixture of cis and trans isomers) which had been prepared in his laboratory a number of years ago. We also obtained a sample of Dr. Hellerman's compound through the courtesy of Dr. Harry Wood of the Cancer Chemotherapy National Service Center (CCNSC); this compound was listed by CCNSC as *trans*-1-amino-1,3-dicarboxycyclopentane (NSC No. 27386), but chromatographic studies carried out in our laboratory showed that the sample contains about equal amounts of the cis and trans isomers, as stated by Dr. Hellerman (personal communication).

We thank Dr. J. W. Wilt for giving us a generous sample of 1-benzamido-2-norbornene (Wilt *et al.*, 1968).

Lactate dehydrogenase and pyruvate kinase were obtained

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