Effect of Immunoglobulin Class and Affinity on the Initiation of Complement-Dependent Damage to Liposomal Model Membranes Sensitized with Dinitrophenylated Phospholipids†

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ABSTRACT: The principal goal of this investigation was to examine some of the factors that determine how much antigen must be incorporated into liposomal model membranes to render them susceptible to immune damage by the classical complement pathway. Liposomes were actively sensitized either with a previously described phospholipid derivative, dinitrophenylphosphatidylethanolamine (I), or a new synthetic analog, dinitrophenylaminocaproylphosphatidylethanolamine (II). Immune damage was assayed by the release of trapped glucose marker in the presence of guinea pig complement and various highly purified rabbit IgG and IgM anti-dinitrophenyl antibodies which were characterized by their association constant (K_0) for ε-Dnp-lysine: high-affinity antibodies had a K_0 of 10^8 l. mol^{-1} and low-affinity antibodies had a K_0 of 10^5 l. mol^{-1}. Less high-affinity IgG antibody was required for glucose release from liposomes sensitized with a constant amount of II than from liposomes sensitized with the same amount of I; conversely, loss of marker initiated by a fixed concentration of high-affinity IgG antibody occurred upon the incorporation of significantly smaller quantities of II than I. Because II is more closely related in structure to ε-Dnp-lysine (the predominant antigenic determinant in the immunogen) than is I, these results therefore support an earlier suggestion that antibody affinity plays an important role. Direct measurement of low- and high-affinity IgG antibody absorption by liposomes sensitized with each of the dinitrophenylated derivatives are also consistent with this conclusion. Antibody affinity is not, however, the only factor involved as indicated by the response of liposomes to low-affinity antibodies belonging to either the IgG or IgM class. In the case of liposomes containing a constant amount of II, glucose release was obtained in the presence of lower concentrations of IgM, as opposed to IgG, antibody; conversely, immune damage in the presence of the IgM antibody required the incorporation of less II than did marker loss initiated by the IgG antibody. These findings suggest the liposomal counterpart of a phenomenon, which was originally encountered in studies of the immune hemolysis of sheep erythrocytes, demonstrating that IgM antibodies are more efficient than IgG antibodies in activating the first component of the complement sequence. Control experiments are described using a mouse myeloma IgA protein which has anti-Dnp specificity; this immunoglobulin does not promote glucose release in the presence of guinea pig serum consistent with the fact that IgA antibodies are unable to activate the classical complement pathway. Interaction of this IgA protein with liposomes containing II was, however, demonstrated by loss of marker which occurred upon subsequent addition of rabbit anti-myeloma protein antiserum; this represents the first case of passive liposomal sensitization to antibody–complement by an antigen that is not an amphipathic lipid.

Previous investigations (see Kinsky (1972) for review) have shown that a variety of amphipathic antigens are effective in the production of immunologically sensitive liposomes that release trapped glucose marker in the presence of an appropriate antibody and complement source. This list includes not only naturally occurring materials (ceramides, lipopolysaccharides, and lipid A) but also synthetic compounds (dinitrophenylated derivatives of phosphatidylethanolamine[1] and lysophosphatidylethanolamine). Although liposomes sensitized with these antigens respond qualitatively in the same way to antibody–complement, important quantitative differences have been observed. For example, a comparison of different ceramide homologs revealed that half-maximal glucose release from sphingomyelin (or lecithin)–cholesterol–dicetyl phosphate liposomes in the presence of excess antibody and complement required the incorporation of one-fifth as much Forssman antigen as globside I (Inoue *et al.*, 1971). More recently, it was shown that an antigen:sphingomyelin ratio of 1:25 was necessary for maximum sensitization with Dnp-PE (Uemura and Kinsky, 1972) whereas an earlier investigation had indicated that maximum sensitization in the case of Forssman antigen occurred at a much lower ratio of 1:250 (Kinsky *et al.*, 1969). The present study was prompted in large measure by our desire to account for these differences.

A clue to the origin of this phenomenon was provided by the realization that the antibodies employed to investigate liposomal sensitization with Dnp-PE belonged exclusively to the IgG class; in contrast, the properties of Forssman sensitized liposomes were examined with commercial rabbit anti-sheep erythrocyte serum in which at least 90% of the anti-Forssman antibodies were of the IgM class. In 1965, Borsos and Rapp reported that IgM anti-Forssman antibodies were far more effective at low concentrations than IgG anti-Forssman antibodies in activating the first component of complement which led subsequently to the immune hemolysis of sheep erythrocytes. Since then, it has been generally believed that a single 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 proxim-

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[1] Abbreviations used are: Dnp-PE, dinitrophenylated phosphatidylethanolamine; Dnp-Cap-PE, dinitrophenylated aminocaproylphosphatidylethanolamine.

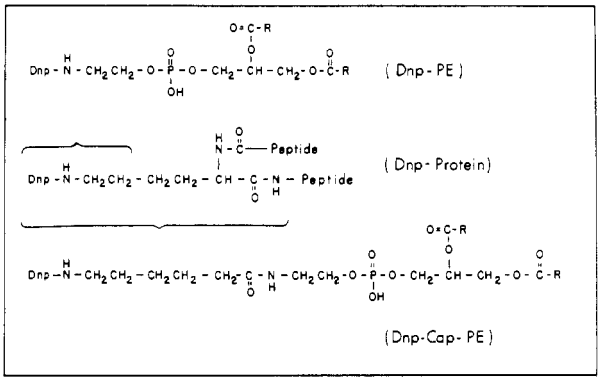

FIGURE 1: Structural comparison of dinitrophenylated protein (used as immunogen in preparation of anti-dinitrophenyl antibodies) with the synthetic dinitrophenylated phospholipid derivatives (used as antigens in the preparation of immunologically sensitive liposomes). See text for full discussion of this figure. Dinitrophenylated protein (Dnp protein) contains ε-Dnp-lysine as the predominant antigenic determinant. The upper bracket encloses the structural region which Dnp protein has in common with dinitrophenylated phosphatidylethanolamine (Dnp-PE); the lower bracket encloses the structural region which Dnp protein has in common with dinitrophenylated aminocaproylphosphatidylethanolamine (Dnp-Cap-PE). R designates long-chain fatty acids esterified at the 1 and 2 positions of glycerophospholipids.

ity are necessary when the immune complex involves IgG antibodies. As a test of this hypothesis, the experiments described in this paper were performed to determine the effect of immunoglobulin class on the amount of antibody and antigen required to produce complement-dependent damage to liposomes.

To assure the validity of such experiments, we considered it essential to compare IgG and IgM immunoglobulins that shared the same immunological specificity. We also regarded it of equal importance to carry out this investigation with immunoglobulins of different classes that possessed the same affinity for the antigenic determinant. Unfortunately, convenient and reliable procedures for measuring the affinity of antibodies for lipid antigens (such as Forssman or lipopolysaccharide) have not yet been established. Appropriate methods (equilibrium dialysis, fluorescence quenching) using water soluble haptenic compounds have, however, been developed for anti-dinitrophenyl immunoglobulins (see, *e.g.*, Eisen and Siskind, 1964) which were therefore employed in these experiments.

The decision to use anti-dinitrophenyl immunoglobulins nevertheless required prior solution of another problem. Eisen and Siskind (1964) have shown that, depending on the time after immunization, rabbits produce anti-dinitrophenyl IgG antibodies whose average intrinsic association constant (K_0) for ε-Dnp-lysine may differ by as much as 3–4 orders of magnitude. Thus, in response to small amounts of immunogen (see below), antibodies elaborated after 2 weeks have a low affinity ($K_0 = 10^5$ l. mol^{-1}) which increases to a much higher affinity ($K_0 = 10^8$ l. mol^{-1}) after 8 weeks. However, under similar conditions, no change in affinity constant was observed in the case of anti-dinitrophenyl IgM antibodies which maintain a low value of 10^5 l. mol^{-1} (Voss and Eisen, 1968). It is important to note that this situation is not confined to rabbits; Voss and Sigel (1972) have recently reported that the association constant of nurse shark anti-dinitrophenyl IgG antibodies for ε-Dnp-lysine increased 100-fold over a 20-month period (from 2×10^5 to 2×10^7 l. mol^{-1}) while the constant for IgM antibodies was not altered (approximately 10^5 l. mol^{-1}). These observations have a direct bearing on the present study because we have previously demonstrated (Uemura and Kinsky, 1972) that liposomes sensitized with Dnp-PE only release trapped glucose marker in the presence of complement when the reaction was initiated with high-affinity IgG anti-dinitrophenyl antibodies ($K_0 = 10^8$ l. mol^{-1}). Accordingly, comparison of liposomal response to IgG and IgM immunoglobulins with the same affinity for dinitrophenyl necessitated the synthesis of a new amphipathic derivative that would react with the low-affinity antibodies.

The design of this derivative took into consideration the fact that the anti-dinitrophenyl immunoglobulins were prepared in rabbits immunized with Dnp-hemocyanin in which ε-Dnp-lysine is the predominant antigenic determinant. Figure 1 illustrates that ε-Dnp-lysine has only a small structural region in common with Dnp-PE; furthermore, Dnp-PE (unlike ε-Dnp-lysine) contains a negatively charged phosphoryl moiety close to the dinitrophenyl group. Failure of the low-affinity antibodies to produce complement-dependent glucose release from Dnp-PE-sensitized liposomes could be explained on this basis (*cf.* Uemura and Kinsky, 1972) and, to examine this hypothesis, we have synthesized Dnp-aminocaproylphosphatidylethanolamine. Figure 1 shows that this phospholipid derivative is more closely related to the antigenic determinant in that it shares with ε-Dnp-lysine a larger structural region (including a peptide bond) and has the phosphoryl moiety farther removed from the dinitrophenyl group. The experiments described below demonstrate that these modifications were sufficient to produce an amphipathic lipid capable of sensitizing liposomes to low-affinity IgG and IgM anti-dinitrophenyl antibodies.

To complete this investigation, we have also examined the effect of an immunoglobulin of the IgA class: specifically, a low-affinity myeloma protein with anti-dinitrophenyl specificity. It will be shown that this protein can function as an antigen to generate passively sensitized liposomes which release glucose marker upon subsequent incubation with anti-myeloma antiserum in the presence of complement.

Experimental Section

Except for some of the immunologic reagents and the synthesis of Dnp-Cap-PE, all of the materials and assay methods employed in this study have been fully described in previous publications as follows.

Lipids Used in the Preparation of Liposomes. See Kinsky *et al.* (1969).

Synthesis and Purification of Dnp-PE, Uemura and Kinsky (1972). The egg phosphatidylethanolamine, which was also required for the preparation of Dnp-Cap-PE (see below), was purchased from Cyclo Chemical, Los Angeles, Calif.

General Procedure for the Generation of Liposomes with Trapped Glucose, Kinsky *et al.* (1969). In this regard, it should be emphasized that the present experiments were performed exclusively with liposomes prepared from a basic lipid mixture containing sphingomyelin, cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.2, respectively.

Specific Procedure for the Generation of Liposomes Sensitized with Dinitrophenylated Phospholipid Derivatives, Uemura and Kinsky (1972). This paper also discusses our rationale for distinguishing between actively and passively sensitized liposomes. In brief, passive sensitization means that the antigen was added after the liposomes (prepared from the basic lipid mixture alone) had been generated; active sensitization means that the antigen was added to the basic lipid mixture

before liposome formation. As in previous publications, liposomes generated under conditions of active sensitization are characterized on the basis of the per cent antigen (relative to sphingomyelin) that was present at the time the model membrane was formed; for example, a liposome preparation actively sensitized with 10% antigen indicates that 100 nmol of either Dnp-PE or Dnp-Cap-PE were incorporated into the basic lipid mixture for every μmol of sphingomyelin.

Immunologic Reagents. Rabbit IgG anti-dinitrophenyl antibodies were generously provided by Dr. H. N. Eisen and Mr. Walter Gray (Department of Microbiology, Washington University School of Medicine, St. Louis, Mo.); rabbit IgM anti-dinitrophenyl antibodies were kindly donated by Dr. Edward Voss (Department of Microbiology, University of Illinois, Urbana, Ill.).[2] Both low-affinity preparations (IgG and IgM) had comparable association constants for ε-Dnp-lysine of 10^5 l. mol^{-1} in contrast to the high-affinity preparations (IgG) which had a constant of 10^8 l. mol^{-1}. Prior to use, the antibodies were dialyzed against Tris-buffered saline as described in Uemura and Kinsky (1972). Antibody concentration was determined, after removal of any insoluble material by centrifugation, from the relative absorbancies at 278 and 360 nm using the empirical formula of Eisen *et al.* (1967).

The IgA myeloma protein (derived from MOPC 315), as well as rabbit antiserum to this protein, were also generous gifts of Dr. H. N. Eisen, Mr. E. S. Simms, and Mr. Walter Gray. Stock solutions of the myeloma protein were prepared by the same procedure employed for the IgG and IgM antibodies. The antiserum was decomplemented by heating at 56° for 30 min and subsequently dialyzed against Tris-buffered saline (Uemura and Kinsky, 1972) to remove endogenous glucose. Preliminary experiments indicated the presence of contaminating anti-dinitrophenyl antibodies and these were removed by absorption with liposomes that had been actively sensitized with 5% Dnp-Cap-PE. For this purpose, 200 μl of liposomes (swollen in 150 mM NaCl) was packed by centrifugation at 27,000*g* for 15 min and the resulting liposomal pellet was dispersed in 1 ml of the dialyzed antiserum. After incubation for 30 min at room temperature, the supernatant solution was recovered following centrifugation as above.

Dialyzed guinea pig serum (Haxby *et al.*, 1968) was used as the source of complement in all experiments.

Coenzymes, Enzymes, Buffers, and Composition of Complete and Incomplete Reagents Used in the Spectrophotometric Assay for Glucose Release from Liposomes, Kinsky *et al.* (1969).

General Procedure for Following Glucose Release in the Presence of Antibody and Complement, Kinsky *et al.* (1969).

Specific Procedure for Following Glucose Release from Liposomes Sensitized with Dinitrophenylated Phospholipid Derivatives, Uemura and Kinsky (1972). This paper describes a modification of the general procedure that was employed to correct for changes in liposomal light scatter occurring as a consequence of agglutination by some antibodies. It, and references cited therein, should also be consulted for details of the calculations involved in expressing the results as "per cent trapped glucose released in 30 min."

[2] According to Drs. Eisen and Voss, these antibodies were purified by established procedures in their laboratories which essentially involved absorption of serum by a Dnp immunoadsorbent or precipitation with a Dnp protein, followed by DEAE and/or molecular sieve chromatography. At least 90% of the protein in these preparations corresponded to specific anti-dinitrophenyl antibodies and immunoelectrophoretic analysis did not reveal contamination of the IgG antibodies by IgM (or *vice versa*).

Miscellaneous. Total phosphate was determined by a minor alteration of the method of Gerlach and Deuticke (1963). The legend to Figure 5 describes the procedure used to determine antibody absorption by liposomes.

Synthesis and Purification of the Dinitrophenylated Derivative of Aminocaproylphosphatidylethanolamine. Dnp-Cap-PE was prepared by acylation of phosphatidylethanolamine with the acid chloride of Dnp-aminocaproic. Dnp-aminocaproic acid (356 mg, 1.2 mmol) was dissolved in 100 ml of redistilled benzene. Thionyl chloride (1 ml, 13.8 mmol) was added and the solution refluxed for 30 min. The reaction mixture was taken to dryness under reduced pressure at 40° and the flask placed in a desiccator under continuous evacuation (0.05 mm) for 1 hr to ensure complete removal of excess thionyl chloride. Phosphatidylethanolamine (0.1 mmol dissolved in 50 ml of redistilled benzene containing 100 mM triethylamine) was added to the dried Dnp-aminocaproyl acid chloride and the mixture stirred at room temperature under a nitrogen atmosphere. After 17 hr, acylation of the phospholipid was complete as indicated by thin-layer analysis of the reaction mixture which showed the absence of any ninhydrin-positive compound.

Dnp-Cap-PE was isolated by chromatography on preparative silica gel plates after initial extraction under conditions in which most of the excess acid chloride of Dnp-aminocaproic was converted to a compound soluble in methanol–water (either the free acid and/or its methyl ester). This was accomplished by taking the reaction mixture to dryness under reduced pressure and transfer of the residue with 125 ml of chloroform to a separatory funnel that contained 100 ml of water and 250 ml of methanol. Subsequent addition of 125 ml of chloroform and 125 ml of water resulted in the separation of two phases (Bligh and Dyer, 1959). The upper methanol–water phase was discarded and the lower chloroform phase was dried; the residue was redissolved in approximately 9 ml of chloroform–methanol (1:1). Equal aliquots were applied to the origin of four silica gel plates (20 × 20 cm; 2 mm thickness; purchased from Brinkmann Instruments Inc., Des Plaines, Ill.) which were developed in a system of chloroform–methanol–water (70:30:5). After migration of the solvent to the top, five distinct yellow bands were visible with approximate R_F values of 0.94, 0.81, 0.67, 0.60, and 0.45. Only one of these bands (R_F of 0.60) contained significant amounts of phosphate and this band was transferred to a chromatography column that contained a bed of Unisil (Clarkson Chemical Co., Williamsport, Pa.) which had been previously washed with chloroform–methanol (1:1). The purpose of this bed was to remove silica gel fines upon elution of the yellow compound with the chloroform–methanol mixture; the eluate was taken to dryness under reduced pressure and the residue redissolved in 25 ml of chloroform.

Phosphate analysis indicated approximately 76% yield (based on the amount of phosphatidylethanolamine added) of a dinitrophenylated phospholipid whose spectrum in absolute ethanol was identical with that of Dnp-aminocaproic and Dnp-PE: maximum absorption at 345 nm with a shoulder at 400 nm. Using a molar extinction coefficient (345 nm) of 17,000 for Dnp-aminocaproic (Little and Donahue, 1968), the spectral data gave a Dnp/phosphate ratio of 1.02 for Dnp-Cap-PE; the corresponding ratio for Dnp-PE was 1.12. These values suggest that both dinitrophenylated phospholipid derivatives are pure which is consistent with the fact that each compound moved as a single yellow spot upon thin-layer chromatography in several solvent systems and no contaminants could be detected with either iodine vapor or ninhydrin

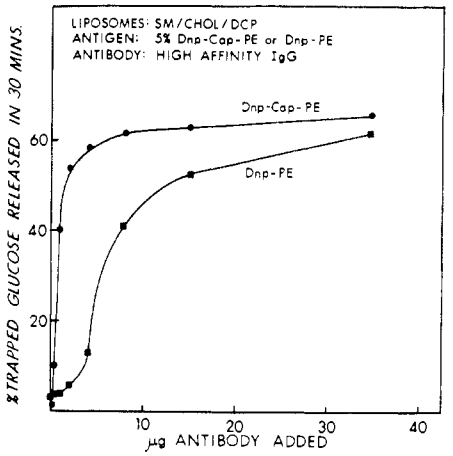

FIGURE 2: Effect of antibody concentration (high-affinity IgG) on glucose release from liposomes sensitized with Dnp-Cap-PE or Dnp-PE. Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing either 5% Dnp-Cap-PE or 5% Dnp-PE. Assays were performed by the standard procedure (Uemura and Kinsky, 1972) in the presence of 125 μl of guinea pig serum and varying amounts of antibody as indicated on the abscissa.

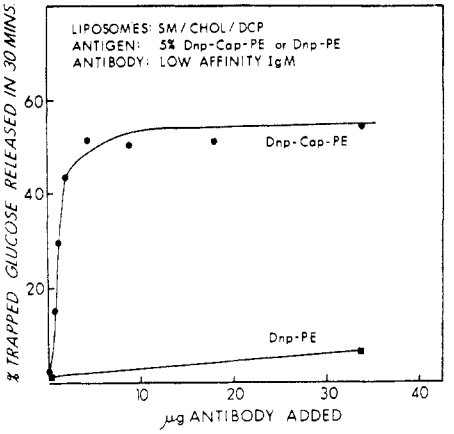

FIGURE 4: Effect of antibody concentration (low-affinity IgM) on glucose release from liposomes sensitized with Dnp-Cap-PE or Dnp-PE. Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing either 5% Dnp-Cap-PE or 5% Dnp-PE. Assays were performed by the standard procedure (Uemura and Kinsky, 1972) in the presence of 125 μl of guinea pig serum and varying amounts of antibody as indicated on the abscissa.

spray. Furthermore, degradation of Dnp-Cap-PE by acid hydrolysis (6 N HCl for 20 hr at 105°) resulted in the formation of one ninhydrin-positive substance and one yellow compound whose chromatographic properties were the same as ethanolamine and Dnp-aminocaproic, respectively; in contrast, similar treatment of Dnp-PE yielded no ninhydrin-reactive material and a single yellow compound identified as Dnp-ethanolamine. These are the expected products from the structures shown in Figure 1.

Results

Comparison of Liposomes Sensitized with Dnp-PE and Dnp-Cap-PE. As demonstrated previously (Uemura and Kinsky, 1972), high-affinity IgG anti-dinitrophenyl antibodies can initiate complement-dependent glucose release from liposomes sensitized with Dnp-PE. It was therefore not surprising to find that Dnp-Cap-PE could function in a similar capacity (Figure 2). Indeed, as illustrated in Figure 2, lower concentrations of antibody were required to produce marker loss from liposomes sensitized with Dnp-Cap-PE than from liposomes sensitized with Dnp-PE.

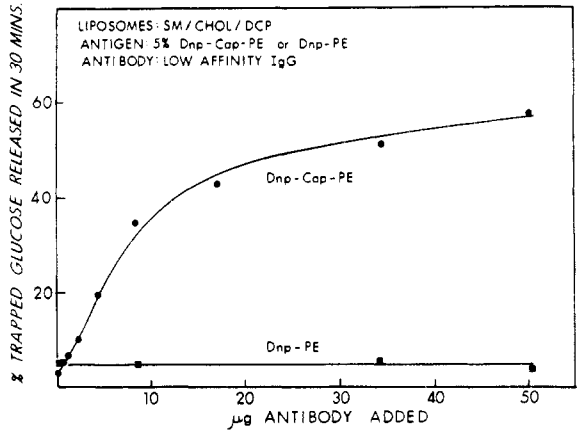

FIGURE 3: Effect of antibody concentration (low-affinity IgG) on glucose release from liposomes sensitized with Dnp-Cap-PE or Dnp-PE. Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing either 5% Dnp-Cap-PE or 5% Dnp-PE. Assays were performed by the standard procedure (Uemura and Kinsky, 1972) in the presence of 125 μl of guinea pig serum and varying amounts of antibody as indicated on the abscissa.

The differential sensitivity of liposomes containing either Dnp-Cap-PE or Dnp-PE was, however, most dramatically revealed by their response in the presence of low-affinity antibody preparations. Figure 3 shows that low-affinity IgG anti-dinitrophenyl antibodies, which cannot cause loss of glucose from Dnp-PE sensitized liposomes, do produce marker release when the liposomes are sensitized with Dnp-Cap-PE. The same phenomenon, in the case of low-affinity IgM anti-dinitrophenyl antibodies, is illustrated in Figure 4.

The preceding results thus support the contention that the inability of the low-affinity antibodies to cause immune damage of liposomes prepared with Dnp-PE is a consequence of the fact that this dinitrophenylated derivative does not have as much structural similarity to the immunogenic determinant (*i.e.*, ε-Dnp-lysine in Dnp-hemocyanin) as does Dnp-Cap-PE. Additional evidence in favor of this argument is provided by experiments which compared the ability of liposomes to bind the low-affinity IgG antibodies. Figure 5 shows that liposomes sensitized with Dnp-Cap-PE absorbed appreciably greater amounts of these antibodies than did liposomes sensitized with Dnp-PE. In regard to the experiment described in Figure 2, it is significant that liposomes sensitized with Dnp-Cap-PE also bound greater amounts of high-affinity IgG anti-dinitrophenyl antibodies than did liposomes sensitized with Dnp-PE (Figure 5). These observations are consistent with earlier evidence (Kinsky *et al.*, 1969; Inoue *et al.*, 1971) indicating that the extent, as well as the rate, of glucose release is dependent on the number of antigen–antibody complexes formed on the model membrane.

Effect of Antibody Concentration on Glucose Release from Liposomes Sensitized with Dnp-Cap-PE. The relative effectiveness of the various anti-dinitrophenyl antibodies can be evaluated from experiments, such as those illustrated in Figures 2–4, on the basis of the concentration required for half-maximal and maximal glucose release from Dnp-Cap-PE-

TABLE I: Antibody Requirement for Glucose Release from Liposomes Sensitized with Dnp-Cap-PE.[a]

Antibody Class	Preparation Affinity	% Dnp-Cap-PE Incorp	Concn (μg/ml) Required for Half-Max. Glucose Release	Concn (μg/ml) Required for Max. Glucose Release
IgG	High	10	0.7	3
IgG	Low	10	3.2	18
IgG	High	5	1.1	8
IgG	Low	5	7.5	30–50[b]
IgM	Low	5	1.2	5

[a] Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing either 5 or 10% Dnp-Cap-PE. The amount of each antibody preparation required for half-maximal and maximal glucose release in the presence of excess complement source (125 μl of guinea pig serum) was determined from titration curves similar to those shown in Figures 2–4. [b] Range.

sensitized liposomes. In making this comparison, it is essential to use liposomes prepared from lipid mixtures that contain the same percentage of dinitrophenylated derivative. Previous investigations with Forssman sensitized liposomes (Kinsky *et al.*, 1969) have indicated that the amount of anti-Forssman antiserum, which produces a given percentage of marker loss, is inversely related to the amount of antigen incorporated into the liposomes. Table I demonstrates a similar phenomenon in the case of purified antibodies; thus, lower concentrations of either high- or low-affinity IgG anti-dinitrophenyl antibodies were necessary for half-maximal and maximal glucose release when the liposomes contained 10%, instead of 5%, Dnp-Cap-PE.

Table I further shows that, regardless of the amount of Dnp-Cap-PE incorporated, high-affinity IgG antibodies were more effective than low-affinity IgG antibodies. However, as regards one of the principal goals of this investigation, the more important finding is that the same percentage of marker loss occurred in the presence of significantly smaller amounts of IgM antibodies as opposed to IgG antibodies with similar affinity. This quantitative difference (6–10-fold on a weight basis) is even greater if the difference in molecular weight of IgG and IgM immunoglobulins is taken into consideration. Using a molecular weight of 150,000 for IgG and 800,000 for IgM, the data in Table I indicate that half-maximal glucose release from liposomes sensitized with 5% Dnp-Cap-PE requires 5×10^{-8} M IgG and 1.5×10^{-9} M IgM antibody; the corresponding values for maximal glucose release are approximately 2.7×10^{-7} and 6.2×10^{-9} M for IgG and IgM, respectively.

Dependence of Glucose Release on the Amount of Antigen Incorporated. The preceding results prompted execution of the converse experiments in which the response of liposomes, sensitized with different amounts of Dnp-Cap-PE, was determined in the presence of a fixed concentration of low affinity IgG and IgM anti-dinitrophenyl antibodies. Figure 6 shows that, when assayed in the presence of excess IgG (38 μg/ml), half-maximal and maximal sensitization of liposomes occurred upon incorporation of 2.1 and 4.0% Dnp-Cap-PE, respectively. In contrast, when the assay was performed in the

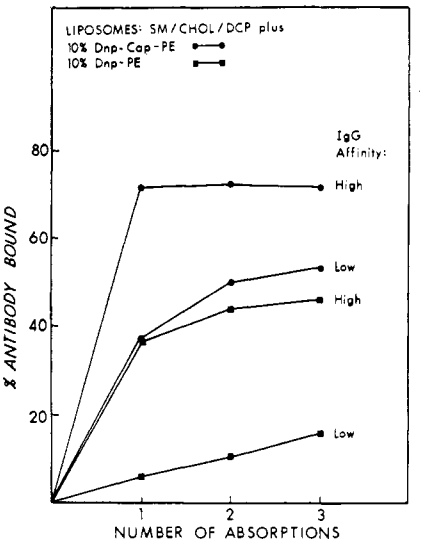

FIGURE 5: Binding of high- and low-affinity IgG antibodies to liposomes sensitized with Dnp-Cap-PE or Dnp-PE. Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing either 10% Dnp-Cap-PE or 10% Dnp-PE. Adsorption was performed as follows. Tubes contained initially 500 μg of the indicated antibody preparations dissolved in 1 ml of 150 mM NaCl–5 mM Tris (pH 7.4); absorbance at 278 and 360 nm was recorded and then 100 μl of the appropriate liposomes were added. After 30 min at room temperature, the tubes were centrifuged for 30 min at 27,000*g* and the absorbance of the supernatant solutions was again measured. This procedure was repeated two more times by the addition of 100 μl of liposomes to the supernatant solutions derived from each previous adsorption. The amount of unbound antibody was calculated from the absorbance values using the formula of Eisen *et al.* (1967) after correction for dilution.

presence of excess IgM (9 μg/ml), only 0.6% antigen incorporation was sufficient for half-maximal sensitization; under these conditions, there was no appreciable change in the amount of Dnp-Cap-PE (4%) which gave maximal sensitization. However, comparison of both low-affinity preparations when present on an equal weight basis (9 μg/ml, corresponding to 6×10^{-8} M IgG and 1.1×10^{-8} M IgM) reveals a marked increase in the amount of this dinitrophenylated phospholipid

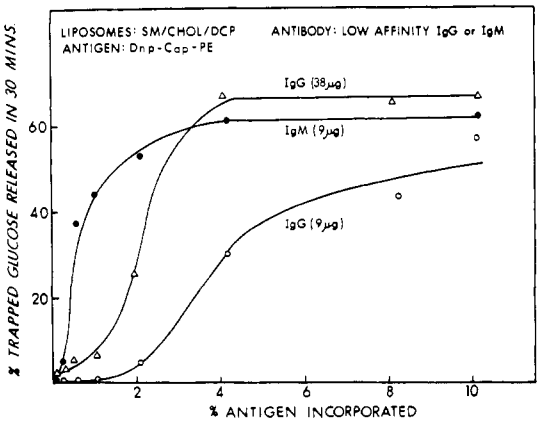

FIGURE 6: Effect of antigen content (Dnp-Cap-PE) on glucose release from liposomes in the presence of low-affinity antibodies (IgG or IgM). Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing varying amounts of Dnp-Cap-PE to give the per cent antigen content indicated on the abscissa. Assays were performed by the standard procedure (Uemura and Kinsky, 1972) in the presence of 125 μl of guinea pig serum and the amounts of low-affinity IgG or IgM antibodies specified in the figure.

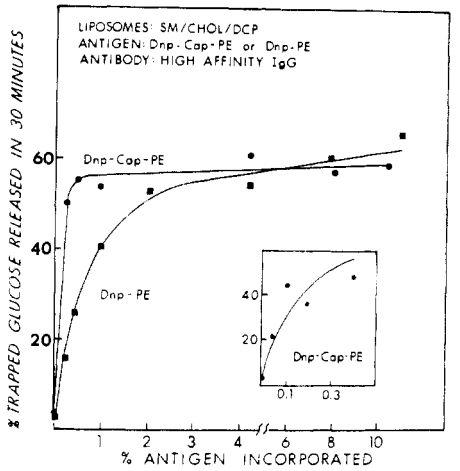

FIGURE 7: Effect of antigen content (Dnp-Cap-PE or Dnp-PE) on glucose release in the presence of high affinity IgG antibody. Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing varying amounts of either Dnp-Cap-PE or Dnp-PE to give the per cent antigen content indicated on the abscissa. Assays were performed by the standard procedure (Uemura and Kinsky, 1972) in the presence of 125 μl of guinea pig serum and 36 μg of high-affinity IgG antibody.

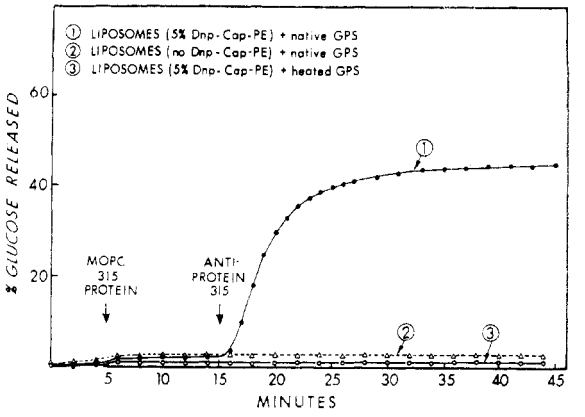

FIGURE 8: Glucose release from liposomes in the presence of protein 315 and anti-protein 315 antiserum. Liposomes were prepared from sphingomyelin–cholesterol–dicetyl phosphate mixtures containing either 0 or 5% Dnp-Cap-PE. Assays were performed by the following modification of the standard procedure. Experimental cuvettes contained 338 μl of Veronal-buffered saline, 500 μl of complete glucose assay reagent, 113 μl of guinea pig serum (native or heated GPS; see figure), and 5 μl of the appropriate liposomes (prepared with and without Dnp-Cap-PE; see figure). Control cuvettes were the same except that 500 μl of incomplete assay reagent was substituted for the complete assay reagent. All cuvettes received 4 μl of protein 315 (10 μg) at 5 min and 40 μl of anti-protein 315 antiserum at 15 min. Absorbance changes at 340 nm were monitored with a Gilford recording spectrophotometer; the per cent glucose released at various times was calculated from the difference in absorbance of the experimental and control cuvettes as described previously (Uemura and Kinsky, 1972).

derivative necessary for maximal sensitization to IgG as opposed to IgM antibodies. Figure 6 also illustrates that it is possible to prepare liposomes whose antigen content is so low (0.5% Dnp-Cap-PE) that they will only undergo complement dependent glucose release in the presence of IgM, but not IgG, antibodies; this finding is especially significant in the context of the current investigation.

Figure 7 shows that even lower levels of Dnp-Cap-PE were sufficient to prepare immunologically responsive liposomes when the assay was carried out in the presence of excess high-affinity IgG antibodies (36 μg/ml): 0.1% for half-maximal sensitization and 0.5% for maximal sensitization. Analogous experiments with Dnp-PE gave corresponding values of 0.5% and approximately 5.0% for half-maximal and maximal sensitization (see also Figure 3 in Uemura and Kinsky, 1972). These findings are entirely consistent with the phenomena described in Figures 2 and 5 indicating that the difference in structure between Dnp-Cap-PE and Dnp-PE has a pronounced effect on the affinity of the various anti-dinitrophenyl antibodies for these dinitrophenylated phospholipid derivatives.

Interaction of Liposomes with an IgA Immunoglobulin. In the course of this study, several types of controls were routinely included to assure that glucose release from Dnp-Cap-PE- and Dnp-PE-sensitized liposomes did indeed reflect a genuine immune response. Thus, essentially no loss of marker (5% or less) was observed when normal rabbit IgG or IgM (*i.e.*, from nonimmunized animals) was substituted for the anti-dinitrophenyl antibodies, or when the guinea pig serum was heated at 56° for 30 min (conditions usually employed to inactivate cytolytic complement activity). These results have not been presented because previous publications have fully documented the requirement for a specific antibody and a source of native complement (see, for example, Kinsky *et al.*, 1969, Inoue *et al.*, 1971, and Kataoka *et al.*, 1971a, 1971b).

Figure 8 describes a different type of control experiment which was made feasible by the availability of a mouse myeloma protein (MOPC 315) of the IgA class capable of binding ε-Dnp-lysine (Eisen *et al.*, 1968). Curve 1 shows that addition of this protein to liposomes (containing Dnp-Cap-PE) did not cause glucose release in the presence of unheated guinea pig serum. This observation is in agreement with the generally accepted view that IgA, in contrast to IgM and most IgG, immunoglobulins are unable to activate the classical complement sequence upon formation of an antigen–antibody complex (see, *e.g.*, Ishizaka *et al.*, 1966). However, in regard to the latter, it was considered necessary to establish that failure of the myeloma protein to produce marker loss was not due to an inability to bind Dnp-Cap-PE. This possibility was rendered unlikely by the demonstration that the subsequent addition of rabbit anti-myeloma protein antiserum did result in glucose release in the presence of native (curve 1) but not heated guinea pig serum (curve 3). Furthermore, curve 2 shows that marker is not released in the presence of myeloma protein, anti-myeloma antiserum, and complement from liposomes prepared without Dnp-Cap-PE. These results indicate that the dinitrophenylated phospholipid is required for the attachment of the myeloma protein to the liposomes and that it is the latter which functions as an antigen effective under conditions of passive sensitization.

This phenomenon was examined in greater detail because it constitutes the first demonstration that a protein may be employed in place of an amphipathic lipid antigen to sensitize liposomes to the action of antibody–complement (see Discussion). The requirement for antibody is illustrated in Figure 9 showing that the extent of glucose release is dependent on the amount of anti-myeloma protein antiserum added; again, it should be noted that the antiserum alone is not able to produce marker loss in the presence of heated guinea pig serum. The requirement for the myeloma protein as antigen is described by the curves in Figure 10 which were derived under different experimental conditions. In curve 1, the liposomes were preincubated with the myeloma protein directly in the assay cuvettes; in curve 2, the liposomes were preincubated with the myeloma protein in test tubes and the liposomes re-

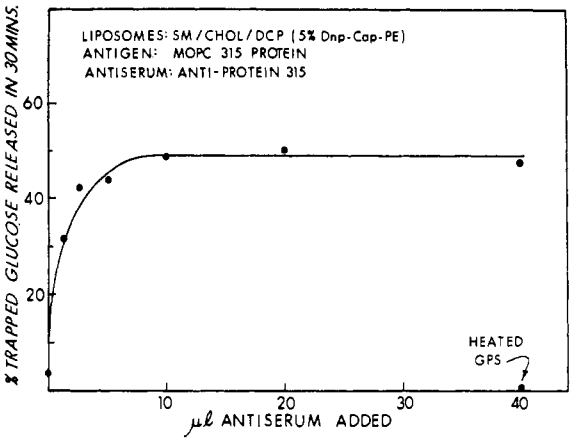

FIGURE 9: Effect of anti-protein 315 antiserum concentration on glucose release from liposomes sensitized with protein 315. Liposomes were prepared from a mixture of sphingomyelin–cholesterol–dicetyl phosphate containing 5% Dnp-Cap-PE. Sensitization was performed by incubation of 200 μl of liposomes with 200 μg of protein 315 dissolved in 320 μl of 150 mM NaCl–5 mM Tris (pH 7.4); after 30 min at 4°, the liposomes were recovered by centrifugation (15 min at 27,000*g*) and resuspended in 200 μl of Veronal-buffered saline. Liposomes were then assayed by the standard procedure (Uemura and Kinsky, 1972) in the presence of 113 μl of guinea pig serum (GPS) and varying amounts of anti-protein 315 antiserum as indicated on the abscissa.

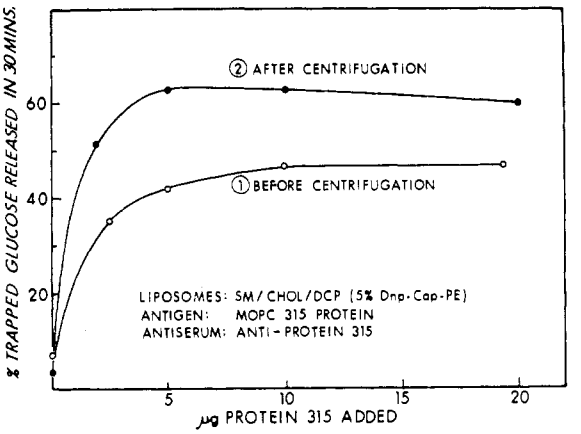

FIGURE 10: Effect of protein 315 concentration on glucose release from liposomes in the presence of anti-protein 315 antiserum. Liposomes were prepared from a mixture of sphingomyelin–cholesterol–dicetyl phosphate containing 5% Dnp-Cap-PE. Sensitization of liposomes with the myeloma protein was performed by two procedures. In the experiment described by curve 1, 5 μl of liposomes were preincubated for 10 min at room temperature in cuvettes which contained 500 μl of complete glucose assay reagent, varying amounts of protein 315 (see below), and sufficient Veronal-buffered saline to give a final volume of 847 μl. Each cuvette then received 113 μl of guinea pig serum and the reaction was initiated by the addition of 40 μl of anti-protein 315 antiserum. In the experiment described by curve 2, 100 μl of liposomes were preincubated for 30 min at 4° in test tubes which contained varying amounts of protein 315 (see below) and sufficient Veronal-buffered saline to give a final volume of 2.26 ml. The liposomes were recovered by centrifugation (15 min at 27,000*g*) and resuspended in 100 μl of Veronal-buffered saline. Five μl of liposomes were then assayed by the standard procedure (Uemura and Kinsky, 1972) in the presence of 113 μl of guinea pig serum and 40 μl of anti-protein 315 antiserum. In both experiments, glucose release was calculated after correction for any changes in light scatter occurring in parallel cuvettes which contained incomplete, instead of complete, assay reagent. These values (ordinate) were plotted against the amounts of protein 315 added directly to the cuvettes (experiment 1) or against the amounts of protein 315 per 5 μl of liposomes added to the test tubes (experiment 2).

covered by centrifugation before addition to the assay cuvettes. As indicated in Figure 10, we have often observed that the centrifuged liposomes release a greater percentage of their trapped glucose than the noncentrifuged liposomes. This suggests that not all of the myeloma protein may be bound to the liposomes, which contain Dnp-Cap-PE, and that the free myeloma protein may function as an inhibitor of complement-dependent immune damage of liposomes initiated by the anti-myeloma serum. A similar phenomenon has been encountered previously (Uemura and Kinsky, 1972) in experiments concerning the passive sensitization of liposomes with the dinitrophenylated derivative of lysophosphatidylethanolamine.

Discussion

The purpose of this investigation was to examine some of the factors which determine how much antigen must be incorporated into liposomes to render these model membranes sensitive to immune damage by the classical complement pathway. This pathway is initiated by the formation of an appropriate antigen–antibody complex on the membrane surface which subsequently activates the first component of the complement system (see, *e.g.*, Müller-Eberhard (1972) for review). Thus, the association constant of the reaction between antibody and antigen, which by definition is a measure of the stability of the immune complex, should have a prominent effect.

Evidence in support of this contention was originally provided by experiments showing that only IgG anti-dinitrophenyl antibodies with high affinity for ε-Dnp-lysine are able to promote glucose release from Dnp-PE sensitized liposomes in the presence of guinea pig complement (Uemura and Kinsky, 1972). The current study has demonstrated that these high-affinity antibodies are also more efficient in causing immune damage of liposomes sensitized with Dnp-Cap-PE as compared to liposomes sensitized with Dnp-PE, *i.e.*, a lower concentration of antibody suffices to produce a given percentage of marker loss (Figure 2 and Table I). Furthermore, we have been able to show that the response of liposomes to a fixed concentration of the high-affinity antibodies requires incorporation of significantly smaller amounts of Dnp-Cap-PE than Dnp-PE (Figure 7). As noted earlier, Dnp-Cap-PE has a closer structural similarity to ε-Dnp-lysine than does Dnp-PE. The preceding findings therefore constitute additional evidence that the affinity between antigen and antibody plays an important role because it is generally accepted that immune complexes are more readily formed with compounds (haptens) whose structure resembles the antigenic determinant against which the antibodies are produced. In this regard, reference should also be made to an earlier study by Fauci *et al.* (1970) who have examined fixation of guinea pig complement by mixtures containing varying concentrations of dinitrophenylated bovine γ-globulin and rabbit IgG anti-dinitrophenyl antibodies with different affinities for ε-Dnp-lysine. They found that less antigen is necessary for complement consumption in the presence of a constant amount of high-, as opposed to low-, affinity antibodies; conversely, smaller amounts of high-, as compared to low-, affinity antibodies were required for complement fixation in the presence of a constant quantity of antigen. Although these experiments were not directly concerned with complement-dependent

damage to liposomes, the results nevertheless provide a precedent for the above conclusion.

The amount of antigen needed to sensitize liposomes to the action of the complement system is, however, not only determined by the affinity of the antibodies but also by the immunoglobulin class to which they belong. We have demonstrated that less Dnp-Cap-PE has to be incorporated into liposomes when the reaction is initiated by IgM, instead of IgG, anti-dinitrophenyl antibodies (Figure 6). Furthermore, the present study has shown that a lower concentration (on either a weight or molar basis) of IgM, as compared to IgG, antibodies will produce a given percentage of glucose release from liposomes which contain a fixed amount of antigen (Figures 3 and 4; Table I). These findings suggest the liposomal counterpart of a phenomenon that was originally described by Borsos and Rapp (1965a) in connection with the immune hemolysis of sheep erythrocytes. They demonstrated that a single molecule of anti-Forssman IgM antibody was sufficient to sensitize these cells to the lytic action of guinea pig complement; this was attributed to the fact that the immune complex on the membrane surface (consisting of Forssman antigen and one molecule of IgM antibody) was capable of activating one molecule of C1 (the first component of the complement system). In the case of IgG anti-Forssman antibody, however, two molecules were necessary to form a site on the erythrocyte surface capable of bindng one molecule of activated C1 (Borsos and Rapp, 1965b). This requirement for doublet immune complex formation with IgG immunoglobulins has since been demonstrated for antibodies of widely different antigenic specificities (see, *e.g.*, Borsos and Rapp, 1965b; Rosse, 1968, 1971). Moreover, this phenomenon is not only restricted to antigens localized on the erythrocyte surface; Cohen (1968) has measured the fixation of guinea pig complement by immune precipitates formed with egg albumin and different ratios of complement fixing and nonfixing rabbit IgG anti-albumin antibodies and also obtained evidence in support of the contention that two "adjacent" antibody molecules are required.

Our experiments with liposomal model membranes confirm and extend the results obtained by Hoyer and Trabold (1971) in their study of the role of erythrocyte antigen site density on immune hemolysis (see also Linscott, 1970). These investigators coupled varying amounts of diazotized radioactive sulfanilic acid to human erythrocytes; the cells were then tested for their complement sensitivity in the presence of either IgG or IgM anti-sulfanilic antibodies prepared in rabbits immunized with diazotized sulfanilic acid coupled to edestin. Depending on the amount of sulfanilic acid bound to the cells, the IgM antibodies were shown to have hemolytic titers that were 10–500-fold greater than the hemolytic titers of the IgG antibodies; also, the threshold antigen site density for hemolysis by the IgM antibodies was approximately fivefold less than that required for hemolysis by the same concentration (weight basis) of IgG antibodies. As noted by Hoyer and Trabold (1971), these experiments were unfortunately performed with cells that did not contain a single antigenic determinant because the coupling procedure leads to the attachment of sulfanilic acid to many different sites on the erythrocyte surface; in addition, the possible contribution of differences in antibody affinity of the IgG and IgM immunoglobulins could not be excluded. However, neither of these objections are applicable to the present experiments employing liposomes sensitized with Dnp-Cap-PE which possess an additional advantage in that the molecular dimensions of each of the amphipathic constituents can be readily determined by monolayer techniques. Accordingly, we believe that these model membranes may provide a unique opportunity for the precise determination of the distance between antigens necessary for the activation of C1 by IgG antibodies.

Finally, it should be noted that the results obtained with the myeloma protein may have some bearing on two aspects of liposomal response which still have not been satisfactorily explained. First, we have long been puzzled by the fact that maximally sensitized liposomes (assayed in the presence of excess antibody and complement source) release approximately six times more glucose than the outermost aqueous compartment would be expected to contain (see, *e.g.*, calculations and discussion in Kinsky, 1972). This implies that an appreciable amount of the released marker probably originates from deeper aqueous compartments as a consequence of damage to internal bilayers. Second, previous studies with antigens that can both actively and passively sensitize liposomes have shown that the conditions employed for sensitization have little effect, *i.e.*, liposomes passively sensitized with either alkali-treated lipopolysaccharide, alkali-treated lipid A, or the dinitrophenylated derivative of lysophosphatidylethanolamine, release as much glucose as liposomes which have been actively sensitized with these materials (Kataoka *et al.*, 1971a, 1971b; Uemura and Kinsky, 1972). These observations have led to the suggestion that damage to internal bilayers need not proceed by the classical complement pathway involving prior formation of an antigen–antibody complex; this argument was based in large measure on the assumption that, in passively sensitized liposomes, the antigen was localized exclusively within the external liposomal bilayer (Uemura and Kinsky, 1972). We have nevertheless entertained some doubts about the validity of this assumption because, up to now, the antigens employed for passive sensitization (*e.g.*, Dnp-lysoPE) are soluble in both polar and nonpolar media; this property of amphipathic substances may provide the basis for a "flip-flop" mechanism (Kornberg and McConnell, 1971) which would lead to the distribution of antigen in all the liposomal bilayers. However, in the case of liposomes passively sensitized with the MOPC 315 protein, it is more difficult to envision a reasonable mechanism by which this protein antigen could become attached to all the liposomal bilayers and, accordingly, our original suggestion may still be applicable.

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Studies on a Honey Bee Sucrase Exhibiting Unusual Kinetics and Transglucolytic Activity†

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ABSTRACT: Honey bee sucrase (invertase) with transglucolytic activity was found to have unusual kinetics. Fructose or *p*-nitrophenol release from the substrates sucrose and *p*-nitrophenyl α-D-glucoside (pNphGlc), respectively, exhibited nonlinear Hofstee plots and the rates became almost first order at high substrate concentrations while glucose production resulted in conventional linear Hofstee plots. The V_m for release of transglucolytic (transferase) products was first order at all substrate concentrations and a vertical Hofstee plot resulted at higher substrate concentrations. When glucose, Tris (tris(hydroxymethyl)aminomethane), methyl α-D-glucoside, fructose, and other compounds were added initially, differing effects on the rates of production of fructose (or *p*-nitrophenol), glucose and transferase products were noted. The results are accounted for by a mechanism which proposes the formation of an enzyme–substrate intermediate which reacts with water for hydrolysis or with acceptor substrates for transglucolysis. The proposed mechanism predicts the absence of a kinetically relevant binding site for the acceptor substrate. In addition to reacting with acceptor substrates such as glucose, methyl α-D-glucoside, fructose, or Tris the acceptor substrate may be the same as the initial substrate. Thus sucrose and pNphGlc can be both initial and acceptor substrates. The nonlinear and first-order kinetics for the production of fructose (or *p*-nitrophenol) and transferase products are ascribed to the effect of this on the V_m values. The identity and possible route of synthesis of the three main transferase compounds were also investigated.

A report on the properties of sucrase (invertase) from insect sources was first published in 1924 (Nelson and Cohn) on the enzyme from honey. More recently some of the properties of sucrase from *Drosophila* (Marzluff, 1969; Huber and Lefebvre, 1971) and other insects (Kawabata *et al.*, 1973) have been reported. The enzyme is also widely distributed outside of the insect kingdom and most forms of the enzyme are known to be transglycolytic (Stanek *et al.*, 1965). The honey enzyme was shown to have transglucolytic activity and analyses of the transferase products have been previously reported (White and Maher, 1953a,b; Gray and Fraenkel, 1953, 1954; Wolf and Ewart, 1955a,b). One form of sucrase which is present in very large amounts in honeybees has been studied in detail and the enzymatic, physical, and chemical properties of this purified enzyme are being reported elsewhere (Huber, 1973). It was found that the kinetics of this highly purified sucrase from honeybees were very unconventional in that nonlinear and first-order Hofstee plots were obtained for the production of some of the products. The unusual kinetics were found to be the result of the combination of hydrolytic and transglucolytic activities of the enzyme. Since sucrases (invertases) from most other sources are also known to possess both hydrolytic and transglycolytic activity (Stanek *et al.*, 1965), it was felt that a study of the rates of formation of the products of the enzyme reaction of the honey bee enzyme should be of considerable interest and of value for application to other systems especially since the kinetic nature of these reactions has not previously been reported. A model for the action of this enzyme is proposed on the basis of the results which were found.

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Experimental Section

Materials. Enzyme grade sucrose and Tris (tris(hydroxymethyl)aminomethane) were obtained from Schwarz/Mann