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Fate of Phospholipids in Liposomal Model Membranes Damaged by Antibody and Complement*

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ABSTRACT: Liposomes, containing either Forssman antigen or globoside I, have been prepared with radioactive [^{32}P]-sphingomyelin and lecithin isolated from rat liver. These liposomes release 50 to 80% of their trapped glucose marker when incubated with the appropriate antiserum and a source of complement. Analysis of the reaction mixtures did not reveal the appearance of any phospholipid degradation product from liposomes which had undergone "immune damage," and approximately 98% of the total radioactivity recovered from thin-layer plates was in the form in which it had been originally incorporated into the liposomal membrane (*i.e.*, as either lecithin or sphingomyelin). The methods employed would have detected a 1% degradation of phospholipid if the radioactive products had behaved chromatographically similar to phosphatidic acid, phosphorylcholine, lysolecithin, glycerylphosphorylcholine, or sphingosylphosphorylcholine. In control experiments, lecithin liposomes were incubated with phospholipase C. Approximately 40–50% of the lipid was degraded when 50% of the trapped marker had been released indicating that measurable amounts of radioactive product (phosphorylcholine) should have been formed if activation of the terminal complement components had led to the generation of an enzymatic activity with properties analogous to exogenous phospholipase C. Subject to the limitations characteristic of all "negative" experiments, the available data are consistent with the hypothesis that complement-dependent membrane damage may not occur by the enzymatic rupture of covalent bonds in phospholipids.

Previous papers have described the preparation of liposomes from either the chloroform-soluble material of sheep erythrocytes (Haxby *et al.*, 1968; Alving *et al.*, 1969) or from simple artificial lipid mixtures containing pure Forssman antigen (Kinsky *et al.*, 1969). These liposomes release trapped glucose marker when incubated with rabbit antisheep erythrocyte serum and a source of complement. The available data suggest that all of the complement components required for maximum damage to natural membranes are also essential for maximum damage to the model membrane (Haxby *et al.*, 1969). Because lipids thus appear to be the "substrate" for complement action, the present investigation was undertaken to determine whether functional impairment of the liposomal membrane is a consequence of phospholipid degradation. Subject to certain experimental limitations, our results indicate that cleavage of covalent bonds in phospholipids does not occur.

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Experimental Design

In these experiments, guinea pig serum and rabbit immune serum were used as the source of complement and antibody, respectively. Because whole serum may be contaminated by some compounds which conceivably could be products of an enzymatic reaction by the terminal complement components (see Figure 8), it was necessary to employ liposomes prepared with radioactive [^{32}P]phospholipids. If release of glucose occurs as a consequence of phospholipid degradation, then it was anticipated that a new radioactive compound should be formed. Under ideal circumstances, appearance of this compound should be dependent on the presence of both native (*i.e.*, unheated) complement and the immune serum. However, further consideration revealed at least 2 reasons for the inadequacy of controls which contained either heat inactivated complement or normal rabbit serum. First, human or guinea pig serum may contain various phospholipase activities (*cf.* legend to Figure 8) and it would be difficult to exclude the possibility that the decomplementation procedure (incubation at 56° for 30 min) would not also inactivate these enzymes. Second, there was no guarantee that normal and immune serum were identical with regard to the absolute activity of phospholipid degradative enzymes.

An alternative experimental approach was, however,

suggested by previous results indicating that antigen must be incorporated into the liposomal structure to confer immune sensitivity to the model membrane (Kinsky *et al.*, 1969). Accordingly, the experiments in the present investigation were designed to determine whether quantitatively greater amounts, if any, of a radioactive product were formed from liposomes containing antigen, compared to liposomes prepared without antigen, when both were incubated with antibody and complement.

Experimental Procedures

Most of the materials, and all of the assay methods, have already been described in a preceding publication (Kinsky *et al.*, 1969). This paper should be consulted for details regarding: (a) sources of chemicals, enzymes, coenzymes, and immunologic reagents (*i.e.*, rabbit antisheep erythrocyte serum and complement); (b) preparation of liposomes with the appropriate antigen (see below); (c) composition of the stock assay reagent and the spectrophotometric procedure for following loss of glucose in the presence of antiserum and complement.

Antigens and Antigloboside I Serum. The antigens employed in this study were Forssman hapten (isolated from sheep erythrocytes) and globoside I (isolated from human erythrocytes). They were the generous gifts of Professor T. Yamakawa and Dr. S. Handa, Faculty of Medicine, University of Tokyo. Forssman hapten (isolated from horse kidney) was also kindly donated by Dr. A. Makita, Department of Biochemistry, Tohoku University, Sendai, Japan. Antiserum to globoside I was prepared in rabbits by intravenous injection of stroma derived from type A human erythrocytes; details of the procedure will be published elsewhere.[1]

Reference Compounds.[2] These were obtained from the following commercial sources: Pierce Chemical Co., Rockford, Ill. (egg phosphatidylcholine, egg lysophosphatidylcholine, beef brain sphingomyelin, phosphatidic acid); Sigma Chemical Co., St. Louis, Mo. (phosphorylcholine, glycerylphosphorylcholine); Applied Science Labs., State College, Pa. (beef phosphatidylserine, beef phosphatidylethanolamine, plant phosphatidylinositol). Sphingosylphosphorylcholine was prepared from beef brain sphingomyelin by minor modification of the procedure of Kaller (1961).

Preparation and Extraction of Radioactive Phospholipids from Rat Liver. [^{32}P]Phosphoric acid was obtained from Mallinckrodt Nuclear, St. Louis, Mo. Prior to use, it was neutralized with NaOH and sufficient NaCl was added to render the solution isotonic (0.154 M). Male albino rats, weighing 150–200 g, were purchased from the Holtzman Co., Madison, Wis. Each rat received by intraperitoneal injection approximately 1.5 ml of the above solution (containing 8 mCi of ^{32}P) and was sacrificed 18 hr later.

Livers from 3 to 6 animals were extracted by a modification of the procedure of Bligh and Dyer (1959). Rat liver (20 g, wet weight) was homogenized with 75 ml of water in a Waring Blendor. The resulting suspension was mixed with 200 ml of methanol, followed by 100 ml of chloroform, and centrifuged for 10 min at 14,000*g*. Each 100 ml of supernatant solution was then thoroughly mixed with 26.3 ml of chloroform and 26.3 ml of water. The phases were separated by centrifugation as above and the upper methanol–water layer (containing essentially all of the inorganic phosphate and nonlipid phosphate compounds) was discarded. The lower chloroform layer was taken to dryness under reduced pressure at *ca.* 40° and the lipid residue was redissolved in sufficient chloroform to give a 1% solution.

Isolation of Crude PC and SM Fractions. Unisil (Clarkson Chemical Co., Williamsport, Pa.) was activated at 110° for approximately 15 hr. Twenty-five grams (suspended in chloroform) was used to make a column *ca.* 3 cm in diameter and 12 cm in height. This column was washed successively with 1000 ml each of chloroform, methanol, and again chloroform. Lipid extract (500 mg in chloroform) was applied to the column and elution was performed according to the following protocol using different mixtures of chloroform and methanol (*N.B.*, fraction number is designated by Roman numeral): I, 1100 ml of 10:1 $CHCl_3$–CH_3OH; II, 1100 ml of 10:1 $CHCl_3$–CH_3OH; III, 800 ml of 7:1 $CHCl_3$–CH_3OH; IV, 600 ml of 5:1 $CHCl_3$–CH_3OH; V, 600 ml of 5:1 $CHCl_3$–CH_3OH; VI, 1200 ml of 5:1 $CHCl_3$–CH_3OH; VII, 700 ml of 2.5:1 $CHCl_3$–CH_3OH; VIII, 700 ml of 2.5:1 $CHCl_3$–CH_3OH; IX, 800 ml of 1.67:1 $CHCl_3$–CH_3OH; X, 800 ml of 1.67:1 $CHCl_3$–CH_3OH.

Thin-layer analysis (see below) of fractions I, II, and III indicated the presence, respectively, of neutral lipids, PE, and a small amount of PC and PS; these fractions were discarded. Fractions IV, V, and VI contained PC with approximately 4% contamination by material which had the relative mobility of PS or PI; these contaminants were subsequently removed as described below. Fraction VII contained primarily PC with a trace amount of SM and was discarded. Chromatographic analysis revealed SM and a trace amount of PC in fraction VIII, SM in fraction IX, and SM plus a small amount of lysoPC in fraction X; SM was isolated from these fractions as described below.

Isolation of Pure PC. Column chromatography on aluminum oxide was used to remove the acidic phospholipids (PS and PI) present in fractions IV, V, and VI. Nonactivated Al_2O_3 (25 g) (Merck and Co., Rahway, N. J.) was suspended in a mixture of 1.5:1 $CHCl_3$–CH_3OH and used to make a column *ca.* 2.5 cm in diameter and 13 cm in height; this was washed with 500 ml of the same solvent mixture followed by 200 ml

[1] Forssman and globoside I (also known as cytolipin K) have the following general structure: *N*-AcGal-Gal-Gal-Glc-ceramide. These two antigens differ in the configuration of the terminal glycosidic linkage which is $\alpha 1 \rightarrow 3$ in Forssman and $\beta 1 \rightarrow 3$ in globoside I. Details concerning the properties of liposomes prepared with globoside I (and analogous liposomes made with galactocerebroside as antigen) will be published separately (K. Inoue, T. Kataoka, and S. C. Kinsky, manuscript in preparation). It should be emphasized in regard to the present investigation that the rate and extent of glucose release from globoside liposomes is dependent on the amount of antigen incorporated, and requires both immune serum and native complement. Similar results have been obtained previously with Forssman liposomes (Kinsky *et al.*, 1969). The experiments described in the text were carried out with liposomes that had been essentially "optimally sensitized" by incorporation of either antigen and incubated with saturating levels of the appropriate immune serum and complement. In this connection, it is important to note that the rabbit anti-Forssman serum does not release marker from globoside I liposomes and, conversely, rabbit antiglobo-side serum has no effect on Forssman liposomes.

[2] The following abbreviations will be used: SM (sphingomyelin); PC and lysoPC (phosphatidylcholine, *i.e.*, lecithin, and lysolecithin, respectively); PE, PS, PI (phosphatidylethanolamine, -serine, and -inositol, respectively); PA (phosphatidic acid); PhC (phosphorylcholine); GPhC and SPhC (glyceryl- and sphingosylphosphorylcholine, respectively).

of chloroform. The above fractions were pooled and evaporated to dryness. Approximately 200 mg of the lipid material (dissolved in chloroform) was applied to the column. The first fraction, eluted with 200 ml of chloroform, was discarded. The second fraction, obtained by passage of 500 ml of 1.5:1 $CHCl_3$–CH_3OH, was filtered to remove fine Al_2O_3 particles, dried, and weighed (*ca.* 160 mg). PC and lysoPC were the only compounds detected on thin-layer plates; the latter arose from breakdown of PC on the column.

Rechromatography of the above material on a Unisil column (*ca.* 2.5 cm in diameter and 9 cm in height) was used to separate PC and lysoPC. After application of *ca.* 140 mg of lipid to the column, the first fraction was obtained by elution with 100 ml of 100:1 $CHCl_3$–CH_3OH. This fraction contained fatty acids and was discarded. The second fraction, eluted with 1000 ml of 5:1 $CHCl_3$–CH_3OH, was evaporated to dryness, redissolved in chloroform, and filtered. The lipid (100 mg) had an approximate specific activity of 10^6 cpm per μmole of phosphate and was characterized as pure PC by one- and two-dimensional thin-layer analysis (see below).

Isolation of Pure SM. Fractions VIII, IX, and X were pooled and evaporated to dryness and the material (not weighed) was dissolved in 7 ml of chloroform. The contaminants, PC and lysoPC, were converted into water-soluble products (fatty acids and GphC) by addition of an equivalent volume of 1 N KOH in methanol and incubation for 1 hr at room temperature. The reaction mixture was adjusted to pH 6.0 with 10 N HCl and 6.7 ml of water was added. After centrifugation (10 min at 14,000*g*), the lower chloroform layer was isolated and evaporated to dryness. The residue was dissolved in 5 ml of chloroform and applied to a Unisil column (*ca.* 1.5 cm in diameter and 7 cm in height) which had been previously washed as described above. The first fraction, obtained by elution with 100 ml of 20:1 $CHCl_3$–CH_3OH, was discarded. The second fraction, eluted with 160 ml of 1.67:1 $CHCl_3$–CH_3OH, was dried under reduced pressure and redissolved in 2 ml of chloroform. This lipid also had an approximate specific activity of 10^6 cpm per μmole of phosphate, and was identified as pure SM by one- and two-dimensional thin-layer analysis as described below.

Liposome Composition. Liposomes were prepared from mixtures which contained the isolated radioactive phospholipid (either PC or SM), cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.22, respectively (see Kinsky *et al.*, (1969) for details). To produce "immunologically sensitive" liposomes, these mixtures were supplemented with sufficient antigen to give the following ratios: 15 μg of Forssman/μmole of PC, 20 μg of globoside/μmole of PC, 31 μg of Forssman/μmole of SM, and 41 μg of globoside/μmole of SM.

Glucose Release in the Presence of Antibody and Complement; Incubation, Lyophilization, and Extraction of Reaction Mixtures. Cuvets (10-mm light path) contained initially 500 μl of stock glucose assay reagent, 353 μl of Veronal-buffered saline (VB^{2+}), 20 μl of commercial rabbit antisheep erythrocyte serum (diluted fivefold with VB^{2+}) or antiglobo-side serum (undiluted), and 122 μl of guinea pig serum (undiluted) as a source of complement. The absorbancy was determined at 340 mμ and then 5 μl of the appropriate liposome preparation (see table and figure legends) was added to start the reaction. After 30-min incubation at room temperature (*ca.* 22°), the absorbancy was again determined. The difference between the final and initial values was used to calculate the "per cent trapped glucose released" by the method which has been described previously (Kinsky *et al.*, 1969). With reference to the latter, it should be pointed out that this paper contains a typographical error in the description of the chloroform–Triton procedure by which total trapped glucose is determined in liposomes. A correction has appeared (Kinsky *et al.*, 1970).

After incubation, the cuvet contents were transferred to 15-ml Corex tubes and lyophilized. The dried material was extracted 3 times by addition of 1.0 ml of chloroform–methanol (1:1), followed by centrifugation in the cold (2°) for 10 min at 20,000*g*. The 3 extracts were combined and stored in tightly sealed containers until they were counted and subjected to chromatographic analysis. In a control experiment to determine the efficacy of this procedure, an aliquot of [^{32}P]lecithin (21,200 cpm) was taken to dryness together with 122 μl of guinea pig serum and 20 μl of antigloboside serum. The first, second, and third extracts contained, respectively, 91.9, 6.9, and 0.6% of the counts initially added for a total recovery of 99.4%. No additional counts appeared in a fourth chloroform–methanol extract and the residue contained essentially no radioactivity (<0.1%).

Glucose Release in the Presence of Phospholipase C; Incubation and Extraction of Reaction Mixtures. Solutions of the enzyme (Type I from *Clostridium welchii;* Sigma Chemical Co.) were prepared just prior to use by dissolving 1 mg in 1 ml of 0.1 M Tris buffer, pH 7.5. The effect of phospholipase C on lecithin liposomes was determined by modification of the above procedure used to measure glucose release in the presence of antibody and complement. Preliminary experiments indicated that higher concentrations of Ca^{2+} ion were required for the action of the enzyme than complement, and the $CaCl_2$ content of the stock glucose assay reagent was therefore increased from 0.15 to 10 mM. Furthermore, phospholipase C—in contrast to the action of antibody and complement—produced a significant increase in liposomal light scatter, and it was thus necessary to include a control cuvet to correct for this variable. The experimental cuvet contained 500 μl of glucose assay reagent (to which 5 μl of 1 M $CaCl_2$ had been added), 5 μl of liposomes, the desired amount of phospholipase C (up to 40 μl), and sufficient VB^{2+} to give a final volume of 1 ml; the control cuvet was similar except that TPN^+ was omitted from the glucose assay reagent. The reaction was initiated by the addition of phospholipase C to both cuvets and, at various times thereafter, the absorbancy at 340 mμ was determined. The per cent trapped glucose released was then calculated from the difference in absorbancies between the experimental and control cuvets.

Simultaneously with the above spectrophotometric assay, a parallel experiment was performed to correlate the extent of glucose release with the amount of liposomal phospholipid degraded by the enzyme. For this purpose we employed essentially the procedure of Kanfer and Brady (1969) which measures the appearance of phosphorylcholine as an increase in water-soluble radioactivity. A series of tubes (15-ml Corex) were set up, each containing a reaction mixture identical with that in the experimental cuvet. At various times after the addition of phospholipase C, the reaction was terminated by the addition of 0.1 ml of a 10% bovine serum albumin solution and, 5 sec later, 0.1 ml of 100% trichloroacetic acid. The tubes were then centrifuged in the cold for 10 min at 12,000*g*. The supernatant solutions were extracted

TABLE I: Glucose Release and Distribution of Radioactivity after Incubation of [^{32}P]Lecithin Liposomes with Antibody and Complement.[a]

Expt No.	Antigen Present in [^{32}P]Lecithin Liposomes	% Trapped Glucose Released	Total cpm in Reaction Mixture	Total cpm in $CHCl_3$–CH_3OH Extract	Total cpm in H_2O Extract
1a	None	4.3	20,500	22,100	40
1b	Forssman	51.2	24,300	27,900	50
2a	None	14.6	16,800	17,900	40
2b	Forssman	60.5	19,500	15,700	0
3a	None	12.2	20,500	23,700	0
3b	Globoside	60.2	23,900	27,300	0
4a	None	19.7	16,800	16,500	20
4b	Globoside	63.9	17,300	18,000	10

[a] Reaction mixtures were set up, assayed for glucose release, and extracted with chloroform–methanol as described in the text. The residue, remaining after the third $CHCl_3$–CH_3OH extraction, was washed once with 0.5 ml of water. Appropriate aliquots of the aqueous and organic extracts were counted in the scintillation fluid recommended by Bruno and Christian (1961). For determination of the total radioactivity in the reaction mixtures, 5 μl of each dialyzed liposome preparation (the same volume which was added to the assay cuvets) was first mixed with 0.5 ml of 1:1 $CHCl_3$–CH_3OH prior to the addition of scintillation fluid. Experiments 1 and 3 were performed with the same preparation of [^{32}P]lecithin; a different preparation was used for experiments 2 and 4.

TABLE II: Glucose Release and Distribution of Radioactivity after Incubation of [^{32}P]Sphingomyelin Liposomes with Antibody and Complement.[a]

Expt No.	Antigen Present in [^{32}P]Sphingomyelin Liposomes	% Trapped Glucose Released	Total cpm in Reaction Mixture	Total cpm in $CHCl_3$–CH_3OH Extract	Total cpm in H_2O Extract
5a	None	14.7	21,500	23,700	60
5b	Forssman	53.3	17,100	21,500	38
6a	None	11.3	21,500	21,400	0
6b	Forssman	83.2	11,900	10,400	0
7a	None	21.6	21,500	18,800	38
7b	Globoside	42.3	21,100	21,300	63
8a	None	11.8	21,500	22,100	0
8b	Globoside	81.9	11,800	11,300	10

[a] Procedure identical with that described in the footnote to Table I. Experiments 5 and 7 were performed with the same preparation of [^{32}P]sphinogmyelin; a different preparation was used for experiments 6 and 8.

twice with 1-ml portions of diethyl ether to remove the trichloroacetic acid, and an appropriate aliquot of the aqueous phase was counted for radioactivity (see below).

Chromatography. Thin-layer chromatography was performed using precoated silica gel plates (20 × 20 cm, 0.25-mm layer without fluorescent indicator; Brinkmann Instruments, Inc., Westbury, N. Y.). Two solvent mixtures were employed for development. System A contained chloroform–methanol–water (70:30:5) and system B contained chloroform–methanol–acetic acid–water (25:15:4:2). System A was generally used for one-dimensional separation. Two-dimensional analysis was performed by initially developing the plates with system A until the solvent front had migrated approximately 18 cm. After drying in a desiccator over P_2O_5, the second separation, at 90° to the first, was carried out as above with system B. Lipids were visualized by several means as appropriate: I_2 vapor, ninhydrin spray, Dragendorf reagent, the phosphate reagent of Dittmer and Lester (1964), or, in the case of radioactive phospholipids, exposure to X-ray film (Kodak Blue Brand) for 5 days.

Counting. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (Model 314) using, except where noted, a solution of "Omnifluor" (0.4% in toluene; New England Nuclear, Boston, Mass.) as scintillation fluid. The "Omni-Bray" mixture recommended by New England Nuclear was employed in the experiment described in Figure 7, and the "XDC" mixture of Bruno and Christian (1961) was used in experiments which involved

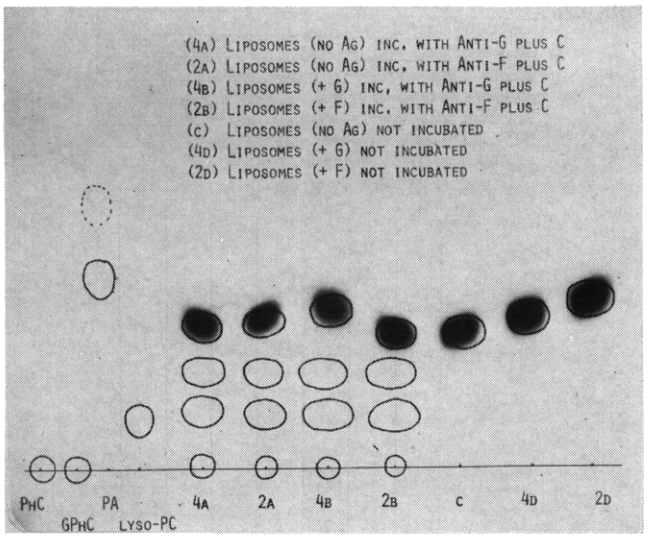

FIGURE 1: Chromatography of chloroform–methanol extracts from reaction mixtures containing [^{32}P]lecithin liposomes. Aliquots of chloroform–methanol extracts, containing *ca.* 3000 cpm (see Figure 3 for exact counts), were chromatographed in one dimension using solvent system A. Extracts 4a, 2a, 4b, and 2b were derived from the corresponding experiments described in Table I. Extracts c, 4d, and 2d were derived from liposomes which had not been incubated with antigloboside serum (anti-G), anti-Forssman serum (anti-F), and complement (C). The liposomes were prepared with either no antigen (Ag), globoside (G), or Forssman (F). Circled areas contained lipid material visualized by I_2 vapor; a reference sample of cold lecithin (not shown) had the same mobility as the radioactive spots. See text for additional details.

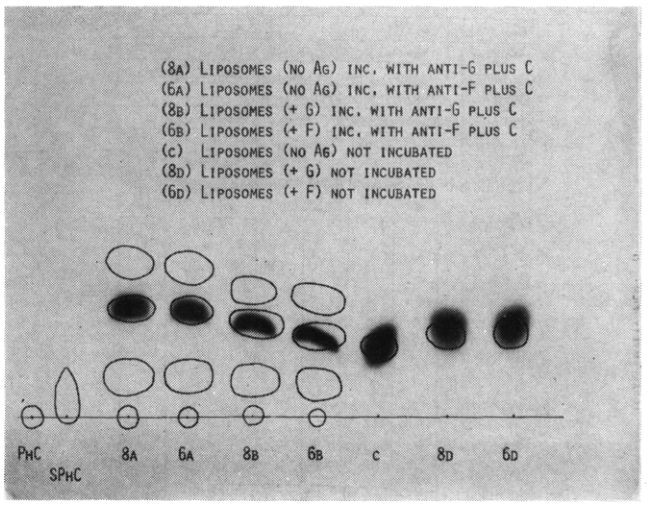

FIGURE 2: Chromatography of chloroform–methanol extracts from reaction mixtures containing [^{32}P]sphingomyelin liposomes. Aliquots of chloroform–methanol extracts, containing *ca.* 3000 cpm (see Figure 4 for exact counts), were chromatographed in one dimension using solvent system A. Extracts 8a, 6a, 8b, and 6b were derived from the corresponding experiments described in Table II. Extracts c, 8d, and 6d were derived from liposomes which had not been incubated with antigloboside serum (anti-G), anti-Forssman serum (anti-F), and complement (C). The liposomes were prepared with either no antigen (Ag), globoside (G), or Forssman (F). Circled areas contained lipid material visualized by I_2 vapor; a reference sample of cold sphingomyelin (not shown) had the same mobility as the radioactive spots. See text for additional details.

comparison of the relative recoveries of radioactivity in chloroform–methanol and aqueous extracts (see legends to Tables I and II).

Results

Glucose Release. Incorporation of antigen (Forssman or globoside) into liposomes prepared with either radioactive lecithin (Table I) or sphingomyelin (Table II) produces a marked increase in the amount of glucose released when these liposomes are incubated with specific antiserum and complement. The experiments cited in Tables I and II also illustrate the variability in liposomal response which we have encountered in the course of the present investigation. Thus, the "enhancement ratio" (defined as the per cent glucose released from liposomes containing antigen/per cent glucose released from liposomes lacking antigen) ranges from 2.0 (expt 7, Table II) to 11.9 (expt 1, Table I). In most cases, incorporation of excess antigen into liposomes made with rat liver lecithin (either cold or radioactive) gives ratios in the range 2–3 (but never less than 2; K. Inoue and S. C. Kinsky, unpublished observations). Subsequent experiments indicated that this was also true of yeast lecithin, and guinea pig liver or kidney lecithin, obtained by the chromatographic procedures described in the preceding section. In contrast, our isolated preparations of rat liver sphingomyelin and egg lecithin, the commercial preparations of beef sphingomyelin and egg lecithin, and synthetic dioleyl- and 1-stearoyl-2-oleyl-PC, generally yielded liposomes with enhancement ratios in the range 4–12. This difference between the various phospholipids was observed with either Forssman or globoside as the antigen, and either guinea pig or human serum as the source of complement (unpublished observations). As indicated in Tables I and II, in some experiments (but not all), the lower enhancement ratios could be attributed to an increased leakage of marker from liposomes prepared without antigen, rather than a decrease in glucose release from liposomes containing antigen.

Distribution of Radioactivity. Incorporation of either Forssman or globoside antigen did not affect the overall distribution of ^{32}P after radioactive lecithin or sphingomyelin liposomes had released glucose in the presence of antibody and complement. Tables I and II show that all of the isotope initially added to the assay cuvets could be recovered in the chloroform–methanol extract regardless of whether the liposomes contained antigen or not. The average recoveries for each of the liposome types were: lecithin (no antigen), 107%; lecithin plus Forssman, 98%; lecithin plus globoside, 109%; sphingomyelin (no antigen), 100%; sphingomyelin plus Forssman, 106%; sphingomyelin plus globoside, 94%. In several experiments (for example, 5b, Table II) the recovery was greater than 100% due to the unavoidable concentration which occurred upon evaporation of some organic solvent during the extraction procedure. Similarly, manipulative losses during extraction may account for the few experiments (for example, 2b, Table I) in which recovery was less than 100%. It should be noted that, in the latter instances, none of the "missing" radioactivity was present in the aqueous extracts.

Chromatographic Analysis. The preceding experiments suggest that highly water-soluble phosphate compounds are not produced when the liposomal membrane has been

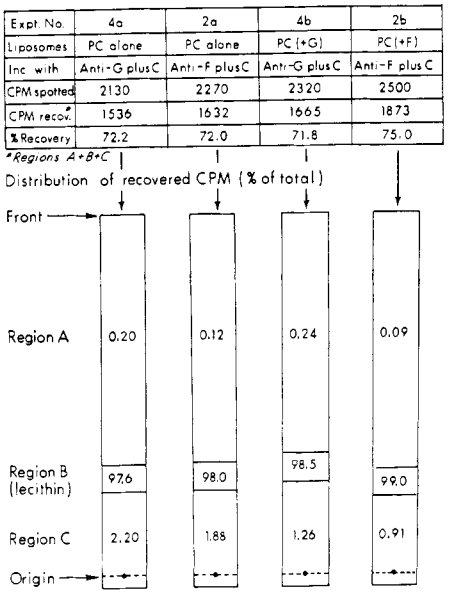

FIGURE 3: Recovery and relative distribution of radioactivity after chromatography of chloroform–methanol extracts from reaction mixtures containing [^{32}P]lecithin liposomes. Appropriate areas from the thin-layer plate shown in Figure 1 were scraped off and counted. Region A corresponds to the area between the top of the radioactive spot to the solvent front, region B contains the visible radioactive area, and region C is the area between the bottom of the radioactive spot and the origin. In calculation of the per cent recovery, correction has been made for radioactive decay occurring between the time that the aliquots were initially spotted and the time that the film was developed (5 days).

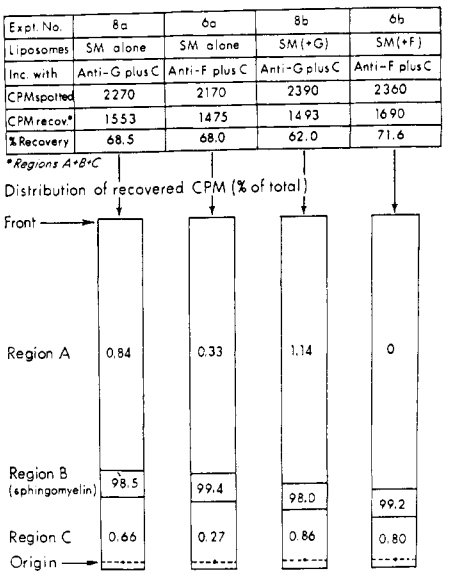

FIGURE 4: Recovery and relative distribution of radioactivity after chromatography of chloroform-methanol extracts from reaction mixtures containing [^{32}P]sphingomyelin liposomes. Procedure was identical with that described in the legend to Figure 3 except that appropriate areas from the thin-layer plate shown in Figure 2 were scraped off and counted.

damaged by complement. This contention was supported by chromatographic analysis of the chloroform–methanol extracts (Figures 1 and 2). In the case of [^{32}P]lecithin liposomes, no radioactive compound (corresponding to phosphorylcholine, glycerylphosphorylcholine, lysolecithin, or phosphatidic acid) could be detected (Figure 1). Analysis of chloroform–methanol extracts from reaction mixtures containing [^{32}P]sphingomyelin liposomes also indicated the absence of any phosphorylcholine and sphingosylphosphorylcholine (Figure 2). When appropriate areas were scraped from the plates and counted, essentially all of the recovered radioactivity was localized in the regions corresponding to either lecithin (Figure 3) or sphingomyelin (Figure 4).

The chloroform–methanol extracts were subsequently subjected to two-dimensional chromatographic analysis. This procedure also did not reveal any new ^{32}P compound as a discrete spot when lecithin liposomes (without and with Forssman or globoside; Figure 5) or sphingomyelin liposomes (without and with the antigens; Figure 6) were incubated with the appropriate antibody and complement under conditions which gave maximum release of trapped glucose marker.

Effect of Phospholipase C on Lecithin Liposomes. The preceding experiments suggest that complement-dependent membrane damage may not occur by any of the common enzymatic reactions involved in the metabolism of phospholipids (*cf.* Figure 8). It must be emphasized that this conclusion is based entirely on "negative" evidence which is subject to the sensitivity of the detection methods used in this investigation. Control experiments indicate that exposure of the plates to X-ray film would have revealed any ^{32}P compound containing 15–30 cpm. Under the present experimental conditions, it should therefore have been possible to detect a 1% degradation of phospholipid upon chromatography of aliquots of the chloroform–methanol extract containing 3000 cpm. It is of course conceivable that covalent bond rupture of less than 1% of the liposomal phospholipids may be adequate to produce the observed amount of glucose release.[3] This possibility was not, however, supported by the

[3] In this connection, it is also very important to emphasize that the process of glucose release from liposomes should not be regarded as a lytic phenomenon. This point came to our attention because a referee, in commenting on the sensitivity of the assay, has noted that "work on complement-dependent lysis of sheep erythrocytes has shown that as few as two bound C-8 molecules are sufficient for lysis of one cell. It is highly unlikely that the lytic effect of a few molecules of C-8 (the eighth component of complement) would be accompanied by detectable amounts of lipid degradation." In our opinion, the above criticism fails to take into consideration a significant difference between erythrocytes and liposomes which may limit the applicability of quantitative data obtained from investigations of sheep erythrocyte lysis to the liposomal system. It is now generally accepted that lysis of sheep erythrocytes proceeds by a colloid–osmotic mechanism which depends on the presence of high concentrations of a large charged macromolecule (hemoglobin) within the single compartment bounded by the cell membrane (see review of Humphrey and Dourmashkin, 1969). Accordingly, it would be expected that only a single "hole," which initially allows cations—but not hemoglobin—to traverse the cell membrane, would eventually lead to lysis. In contrast, the liposomes used in the present investigation are a multicompartment system, *i.e.*, an alternating array of lipid bilayers separated by aqueous regions containing the trapped glucose marker. These liposomes do not contain any large macromolecule in solution. Therefore, colloid–osmotic lysis, analogous to that which occurs with erythrocytes is not possible, and we consider it highly unlikely that a single (or few) holes could account for the observed levels of glucose release. The above discussion should, of course, not be taken to imply that there is a difference in the mechanism by which complement produces lesions that permit ions to enter and leave the damaged erythrocyte membrane, and lesions that facilitate glucose release from liposomes.

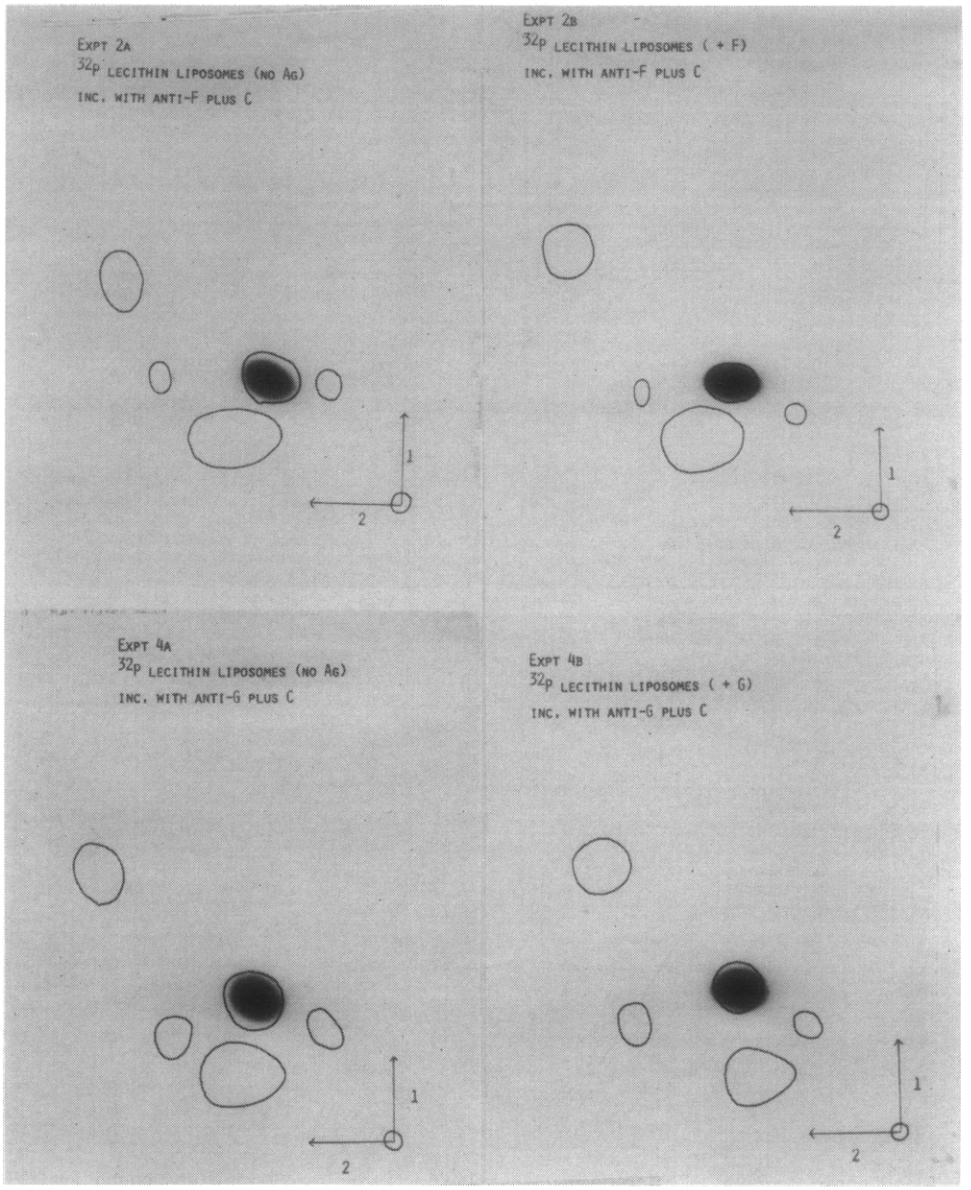

FIGURE 5: Two-dimensional chromatography of chloroform–methanol extracts from reaction mixtures containing [^{32}P]lecithin liposomes prepared with either no antigen (Ag), Forssman (F), or globoside (G). The extracts were derived from experiments 2a and 2b (top row), and 4a and 4b (bottom row), described in Table I. Aliquots, containing *ca.* 3000 cpm, were spotted at the origin (intersection of the arrows). The arrows labeled 1 and 2 refer to the directions in which the plates were developed with solvent systems A and B, respectively (see Experimental Procedures). Circled areas contained material visualized by I_2 vapor.

experiment described in Figure 7 in which the ability of phospholipase C to release glucose from radioactive lecithin liposomes was correlated with the extent of lipid degradation. The results, obtained with 2 different concentrations of enzyme, indicate that release of 50% of the trapped glucose marker (the extent usually observed with excess antibody and complement) is accompanied by 40–50% degradation of the phospholipid. Similar experiments (not reported) with phospholipase A have shown that at least 30% of the lecithin is degraded when the liposomes have released 50% of their trapped glucose.

Discussion

Liposomal model membranes, which apparently respond like natural cell membranes during immune lysis, can be made from mixtures containing only 4 lipids of known structure: a phospholipid, a sterol, a charged amphiphile, and an amphipathic antigen. Such liposomes provide a means to determine whether complement-dependent membrane damage necessarily involves enzymatic degradation of membrane lipids.

A priori, it would seem unlikely that rupture of covalent bonds in either the sterol, charged amphiphile, or antigen, occurs for the following reasons. First, Gram-negative bacteria (which lack sterols) are susceptible to immune lysis in a reaction which requires all the complement components necessary for immune hemolysis of sheep erythrocytes (Goldman *et al.*, 1969) and glucose release from sensitized liposomes (Haxby *et al.*, 1969). Second, dicetyl phosphate

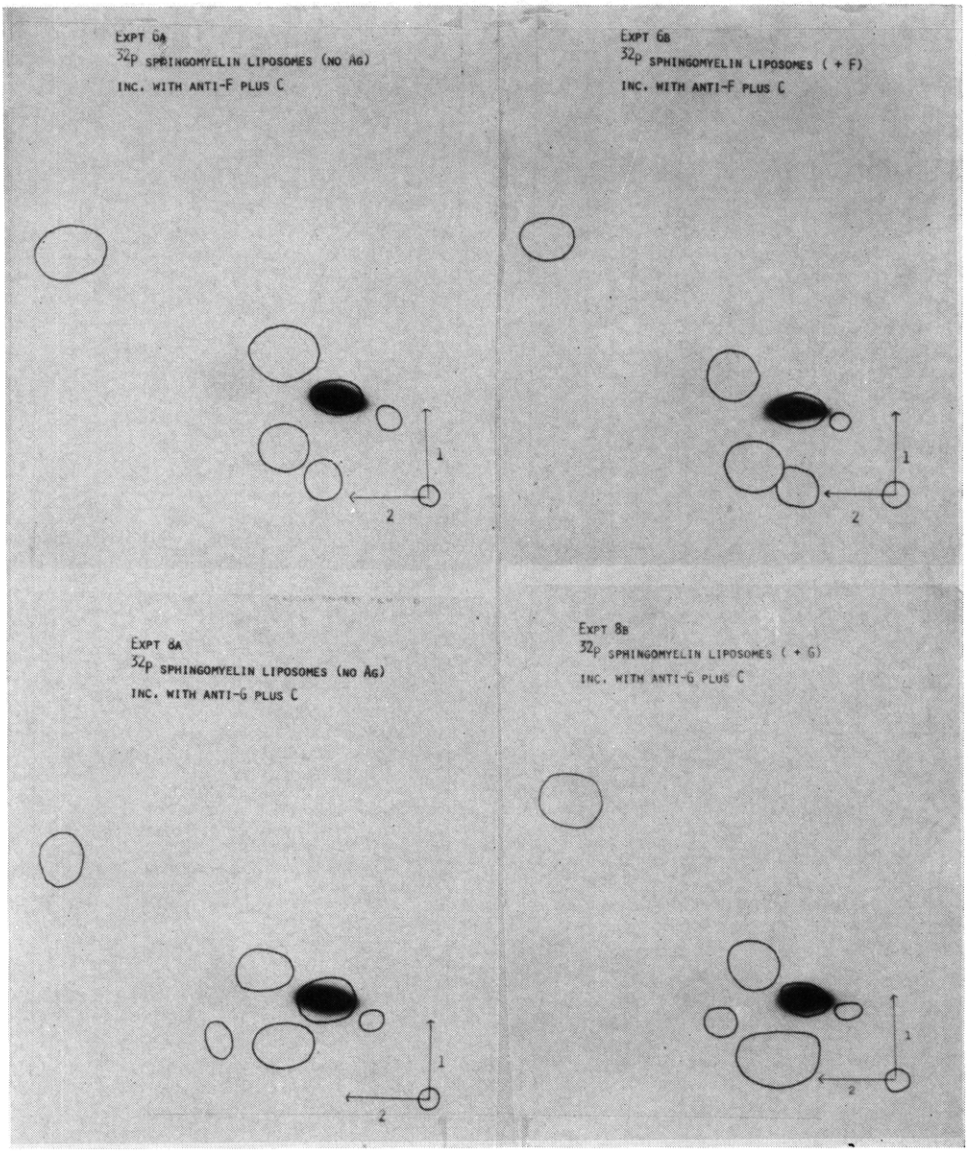

FIGURE 6: Two-dimensional chromatography of chloroform–methanol extracts from reaction mixtures containing [^{32}P]sphingomyelin liposomes prepared with either no antigen (Ag), Forssman (F), or globoside (G). Procedure was identical with that indicated in the legend to Figure 5 except that the extracts were derived from experiments 6a and 6b (top row), and 8a and 8b (bottom row), described in Table II.

(DCP) and stearylamine (SA) can serve equally well as the charged amphiphile for the production of immunologically responsive liposomes (Kinsky *et al.*, 1969). The resultant liposomes are either negatively (DCP) or positively (SA) charged, and it is improbable that an enzyme of sufficiently broad specificity, capable of cleaving both these substrates, would be generated by the sequential action of the complement components. Furthermore, DCP and SA are not present in natural cell membranes. Third, antibodies directed against membrane antigens of widely different structure (*e.g.*, proteins, ceramide derivatives such as Forssman or globoside I, lipoplysaccharides from Gram-negative bacteria) can be used to sensitize cells to the lytic action of complement and, in this case also, it would appear unlikely that a single enzyme could attack these substrates.

In contrast to the above, phospholipids occur in all natural membranes that are susceptible to immune lysis. Accordingly, in the present investigation, we have examined the fate of 2 phospholipids in liposomes which have been damaged by complement. By use of [^{32}P]lecithin and [^{32}P]sphingomyelin, it was possible to show that essentially all of the label could be recovered in the form in which it was originally incorporated into the liposomal membrane. No new radioactive products (corresponding to those indicated by an asterisk in Figure 8) were found in reaction mixtures containing liposomes which had released a significant amount of trapped glucose marker.

The above observations are, however, at variance with some recent experiments of Smith and Becker (1968). They reported that immune hemolysis of sheep erythrocytes was accompanied by a small increase in the amount of titratable free fatty acids with a concomitant decrease in phosphatidylserine and, under some conditions, lecithin, or phosphatidylethanolamine. A possible association of phospholipase A activity with activation of the complement system was

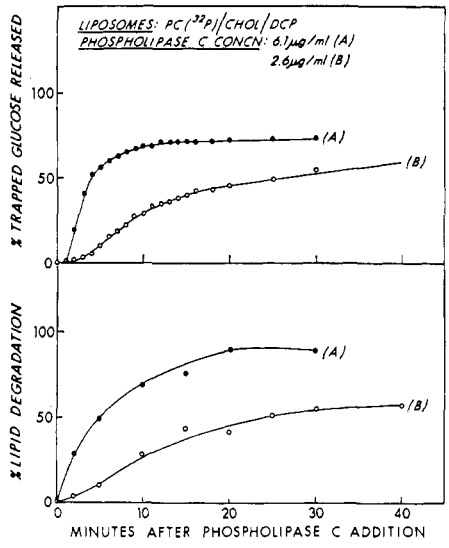

FIGURE 7: Effect of phospholipase C on lecithin liposomes. The experiment was performed as described in the text with the concentrations of phospholipase C indicated in the Figure; 5 μl of the radioactive liposome preparation initially contained 8052 cpm and the per cent lipid degradation was calculated from the amount of radioactivity, due to the formation of phosphorylcholine, which appeared in the aqueous phase.

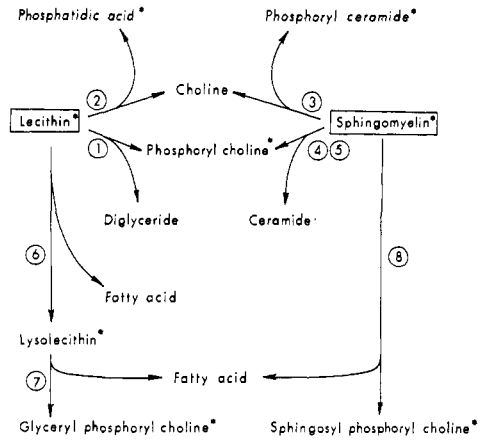

FIGURE 8: The diagram illustrates the possible radioactive products (*) which could be formed from [^{32}P]lecithin or [^{32}P]sphingomyelin if activation of the terminal complement components results in the generation of the following known or "hypothetical" enzyme activities: (1) phospholipase C; (2) phospholipase D; (3) "a phospholipase D also active on sphingomyelin;" (4) sphingomyelinase; (5) "a phospholipase C also active on sphingomyelin;" (6) phospholipase A or B; (7) phospholipase B or lysophospholipase; (8) "sphingomyelinamidase." Immunologically sensitive liposomes can be made with either sphingomyelin or lecithin and, therefore (3) or (5) might be anticipated because these postulated enzymes could act upon both phospholipids. However, the expected products (*e.g.*, PA and PhC) were not detected when the appropriate liposomes were incubated with antibody and complement (see Results).

suggested by the observation that there was no change in the amount of sphingomyelin; the latter is not a substrate for the enzyme but is the major phospholipid in sheep erythrocyte membranes. However, phospholipase A generation seems unlikely in view of the experiments showing that sphingomyelin can be used to prepare "immunologically sensitive" liposomes. Furthermore, Smith and Becker were not able to detect any lysophospholipids and, occasionally, observed an increase in free fatty acids when heated guinea pig serum (hemolytically inactive) was substituted for native serum as a source of complement. In our opinion, the significance of their observations must await more conclusive evidence showing that (a) free fatty acid production is a general phenomenon accompanying immune cytolysis of other cell types in addition to erythrocytes; (b) the free fatty acids were indeed derived from membrane lipids; and (c) lipid degradation occurs before, and not after, immune lysis (the likelihood of some "postlytic" free fatty acid production is indicated by Figure 4 in their paper).

The *available* data, obtained with the liposomal system, are consistent with the earlier suggestion that complement-dependent glucose release from liposomes (and, by implication, immune lysis) probably does not involve rupture of covalent bonds in membrane lipids. A current working hypothesis is that hydrophobic regions within the terminal complement components (components 8 and/or 9) are exposed upon activation by the sequential reactions of the complement cascade, and that interaction of these regions with membranes diminishes the nonpolar intermolecular attractive forces between lipids. In this regard, the mechanism by which complement may produce lysis would appear to resemble more closely the action of the polyene antibiotics (reviewed in Kinsky, 1970), or streptolysins and bee venom mellitin (Sessa *et al.*, 1969), rather than direct action of an enzyme upon the membrane.

It is of course necessary to stress the tentative nature of the above conclusion because it is based on the negative results obtained in the present investigation, and there are inherent limitations in the control experiments. For example, it is possible that an enzyme is generated from the terminal complement components whose action on lecithin or sphingomyelin does not produce a product with chromatographic properties different from the parent phospholipid. Furthermore, although the results obtained with phospholipase C (Figure 7) suggest that appreciable lecithin degradation must occur in order to obtain the levels of glucose release observed in the presence of antibody and complement, we can not exclude the possibility that the properties of an enzyme generated from complement components *in situ* may not be the same as exogenous phospholipase C. In this connection, it seems appropriate to mention a significant difference between liposomes prepared from lecithin and liposomes made with sphingomyelin.[4] In the latter case, we have found that high concentrations of phospholipase C do not cause glucose release under conditions which produce 50% loss of marker in the presence of antibody and complement. The inability of phospholipase C to promote glucose release was consistent with the finding that sphingomyelin had not been degraded when it was present in the form of liposomes. The sphingomyelin could, however, serve as substrate for the enzyme as indicated by the fact that phosphorylcholine was produced when the reaction mixtures were lyophilized and resuspended in the presence of water saturated with chloroform (*i.e.*, conditions which destroy liposomal structure).

[4] Details of these experiments will be published elsewhere (K. Inoue, C. B. Kinsky, and S. C. Kinsky, manuscript in preparation).

Thus, while these results would support the possibility that any phospholipase C derived from complement may differ from the exogenous enzyme as regards accessibility to the sphingomyelin substrate, in our opinion, the same observations may be used to argue that phospholipase C activity is not produced upon activation of the terminal complement components.

Acknowledgments

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25,26-Dihydroxycholecalciferol, a Metabolite of Vitamin D_3 with Intestinal Calcium Transport Activity*

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ABSTRACT: A metabolite of vitamin D_3 (40 μg) has been isolated in pure form from the plasma of eight pigs given 250,000 IU of vitamin D_3/day for 28 days. It has been unequivocally identified as 25,26-dihydroxycholecalciferol by means of mass spectrometry and ultraviolet absorption spectra. This metabolite has some activity in intestinal calcium transport, but is virtually inactive in the cure of rickets and in the mobilization of bone mineral in rats.

Since the successful identification of 25-HCC[1] (Blunt *et al.*, 1968a) and 25-HEC (Suda *et al.*, 1969), it has been strongly suggested that all vitamin D compounds must be hydroxylated in the 25 position before they are active. Both 25-HCC and 25-HEC were 1.4–1.5 times more active than vitamin D_3 or D_2 in curing rickets in rats (Blunt *et al.*, 1968b; Suda *et al.*, 1969). Not only were they more active than their parent vitamins in the stimulation of intestinal calcium transport and in the mobilization of bone mineral, but they acted more rapidly (Blunt *et al.*, 1968b; Suda *et al.*, 1970a). In addition, 25-HCC was effective in isolated target organs while vitamin D_3 itself was without effect (Olson and DeLuca, 1969; Trummel *et al.*, 1969). Therefore, 25-hydroxy D vitamins seemed to be at least the circulating or hormonal active forms of the vitamins (DeLuca, 1969).

Recent studies on the metabolism of [^3H]vitamin D_3 have demonstrated that other metabolites, more polar chromatographically than either vitamin D_3 or 25-HCC, are present in certain tissues and blood following administration of physiological doses of the radioactive vitamin D or 25-HCC (Ponchon and DeLuca, 1969; Haussler *et al.*, 1968; Lawson

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[1] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 25-HEC, 25-hydroxyergocalciferol; 21,25-DHCC, 21,25-dihydroxycholecalciferol; 25,26-DHCC, 25,26-dihydroxycholecalciferol.