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SELECTIVE RELEASE OF NON-ELECTROLYTES FROM LIPOSOMES UPON PERTURBATION OF BILAYERS BY TEMPERATURE CHANGE OR POLYENE ANTIBIOTICS

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Summary

A new system for assaying the permeability characteristics of liposomes was established using Amicon cells equipped with a membrane filter (pore size, 0.3 μm). In this system, damage of liposomes during the assay procedure was negligible. Changes in permeability to non-electrolytes, such as glucose (M_r 180), sucrose (M_r 342), inulin (M_r 5000) and dextran (M_r 75 000), induced by perturbation of the bilayers were examined with this system.

The following results were obtained on the barrier properties of multilamellar liposomes modified by various treatments.

1. Amphotericin B and nystatin did not cause any change in permeability to glucose of egg yolk phosphatidylcholine liposomes prepared in physiological saline and containing trace amounts of radioactive markers in their aqueous compartments. Both antibiotics, however, induced nonspecific release of glucose, sucrose, inulin and dextran from liposomes that contained 0.3 M glucose in their aqueous compartments. These antibiotics first seem to form pores through which small ions can permeate; Na^+ and Cl^- can enter the liposomes through these pores, whereas glucose in the liposomes cannot pass out. As a result, the liposomes become swollen with consequent severe disruption of their membranes.

2. Filipin and digitonin disrupted the membrane structures, resulting in release of large molecules such as dextran even in the absence of an osmotic mechanism.

3. Perturbation of the phase equilibrium by temperature change resulted in formation of 'pores'. The penetration of cations and anions through these 'pores' was apparently much faster than that of glucose, since when liposomes swollen in 0.3 M glucose were incubated in salt solution they were disrupted

by an osmotic mechanism releasing not only glucose but also dextran. Most of the 'pores' were not large enough to allow passage of large non-electrolytes, such as inulin and dextran, since no appreciable amounts of these markers were released from liposomes under conditions where there should be no osmotic gradient.

4. At a temperature well above the phase transition temperature, egg yolk phosphatidylcholine liposomes exhibited specific release of glucose. This process did not involve an osmotic gradient, indicating that it was mainly due to diffusion of the solutes through the bilayers.

Introduction

Numerous methods are available for measuring the permeability of liposomes. Most of these methods involve measurement of the rate of leakage of a marker permeant from within the liposomes into a large sink of known volume (see review of Bangham et al. [1]). Since it is not practical to centrifuge liposomes out of solution, dialysis techniques have generally been used. The dialysis technique chosen, however, depends on the rate of permeation of markers through the dialysis membrane, and so this technique cannot be used to measure the permeability of liposomes to rather large molecules such as proteins. A Sephadex column can also be used for separating liposomes from solution. However, neither method is suitable for following the kinetics of permeation of markers, since the procedures involved are complicated and rather slow. Thus, other methods were required for rapid accurate measurements of the permeability of liposomes to various markers.

Recently, an assay technique using a membrane filter was developed [2,3]. In this method the suspension of reconstituted liposomes is filtered through a membrane filter, and the permeability of the liposomes is determined by measuring the amount of radioactive marker retained in the liposomes on the filter. However, the liposomes on the filter must be washed several times, and they may be damaged during this procedure. In fact, we found that leakage of markers measured with membrane filters was always greater than that measured with other assay systems, such as enzyme assay [4].

The present paper describes the establishment of a new system for assaying the permeability characteristics of liposomes using an Amicon cell equipped with a membrane filter. By this method, we obtain information on the size of 'lesions' formed in liposomal membranes as a result of perturbation of the bilayers.

Materials and Methods

Chemicals. Chemicals were obtained from the following companies: Oriental Yeast Company, Tokyo, Japan (hexokinase, glucose-6-phosphate dehydrogenase and NADP); Sigma Chemical Company, St. Louis, MO, U.S.A. (cholesterol, β,γ-dipalmitoyl-DL-α-glycerophosphocholine and dicetyl phosphate); Merck, Darmstadt, F.R.G. (digitonin); Japan Millipore Ltd., Tokyo, Japan (membrane filters); Radiochemical Centre, Amersham, U.K. (D-[6-^{3}H]-

glucose, [^{3}H]inulin and [4-^{14}C]cholesterol); New England Nuclear, Boston, MA, U.S.A. ([*carboxyl*-^{14}C]dextran carboxyl and D-[U-^{14}C]sucrose). Egg yolk phosphatidylcholine was prepared by chromatography on Aluminum Oxide Neutral and Unisil. All lipid preparations gave a single spot on silica gel thin-layer chromatography. Nystatin (5600 units/mg) and amphotericin B (986 μg/mg) were kindly donated from Squibb Japan Inc., Tokyo, Japan. Filipin was a gift from Up John Co., Kalamazoo, MI, U.S.A.

Preparation of liposomes. Multilamellar liposomes were prepared as described previously [5]. Sonicated liposomes were prepared by sonicating multilamellar liposome preparations for 30 min in a Branson Sonifier (Model W-185). The liposomes consisted of egg yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 10/1/5), or dipalmitoylglycerophosphocholine and dicetyl phosphate (molar ratio, 10/1). The dried lipid film in a 10 ml tube was swollen in 0.2 ml of 0.3 M glucose containing a trace of [^{14}C]dextran (M_r 75 000), [^{3}H]inulin (M_r 5000), [^{14}C]sucrose (M_r 342) or [^{3}H]glucose (M_r 180) as marker. Lipids were also swollen in 0.15 M NaCl instead of 0.3 M glucose. Untrapped glucose and sucrose were removed by dialyzing the preparations at room temperature against isotonic salt solution for more than 2 h. Untrapped inulin and dextran were removed from the liposome preparations by floating the liposomes in 0.3 M sucrose, by a modification of the method of Alving and Kinsky [6]. For this, 300 μl of liposome preparation was layered on 0.3 M sucrose and centrifuged at 100 000 × *g* for 10 min. The supernatant solution underneath the floating layer of liposomes was carefully withdrawn. Then fresh 0.3 M sucrose was added, and after mixing the tubes were recentrifuged. This process of floatation and washing of the liposomes was repeated twice more, and finally the liposomes were suspended in about 300 μl of 0.15 M NaCl.

Calorimetric study. The phase transition temperature was determined by differential scanning calorimetry (Daini Seikosha SSC-544 apparatus).

Measurements of liposomal permeability. The equipment used is illustrated in Fig. 1. An aliquot of liposome suspension (2 ml) containing 100 nmol of phosphatidylcholine was poured into the ultrafiltration cell (Amicon Model 12, Amicon Corp., MA, U.S.A.), equipped with a Millipore membrane filter (type PH, pore size, 0.3 μm). Gradual increase of pressure of the N_2 gas forced the fluid in the cell through the membrane, and a pressure giving a suitable rate of filtration (2 ml/min) was chosen. Throughout the experiment, the fluid inside the cell was stirred gently and constantly to prevent precipitation of liposomes onto the membrane surface with consequent damage of liposomal membranes. Filtration was stopped after about 30 s when about half the solution had passed through the filter, and about 1 ml of solution remained in the cell. It took about 30 s to complete this filtration process. Then the radioactivity in aliquots (0.5 ml) of the filtrate was counted in a liquid scintillation spectrometer (Packard Model 3320). In some experiments, glucose release from liposomes was assayed enzymically with hexokinase and glucose-6-phosphate dehydrogenase, by a modification of the method of Kinsky et al. [4]. The amounts of NADPH generated were determined fluorophotometrically with a Recording Spectrofluorophotometer (Shimadzu Seisakusho RF-501 apparatus).

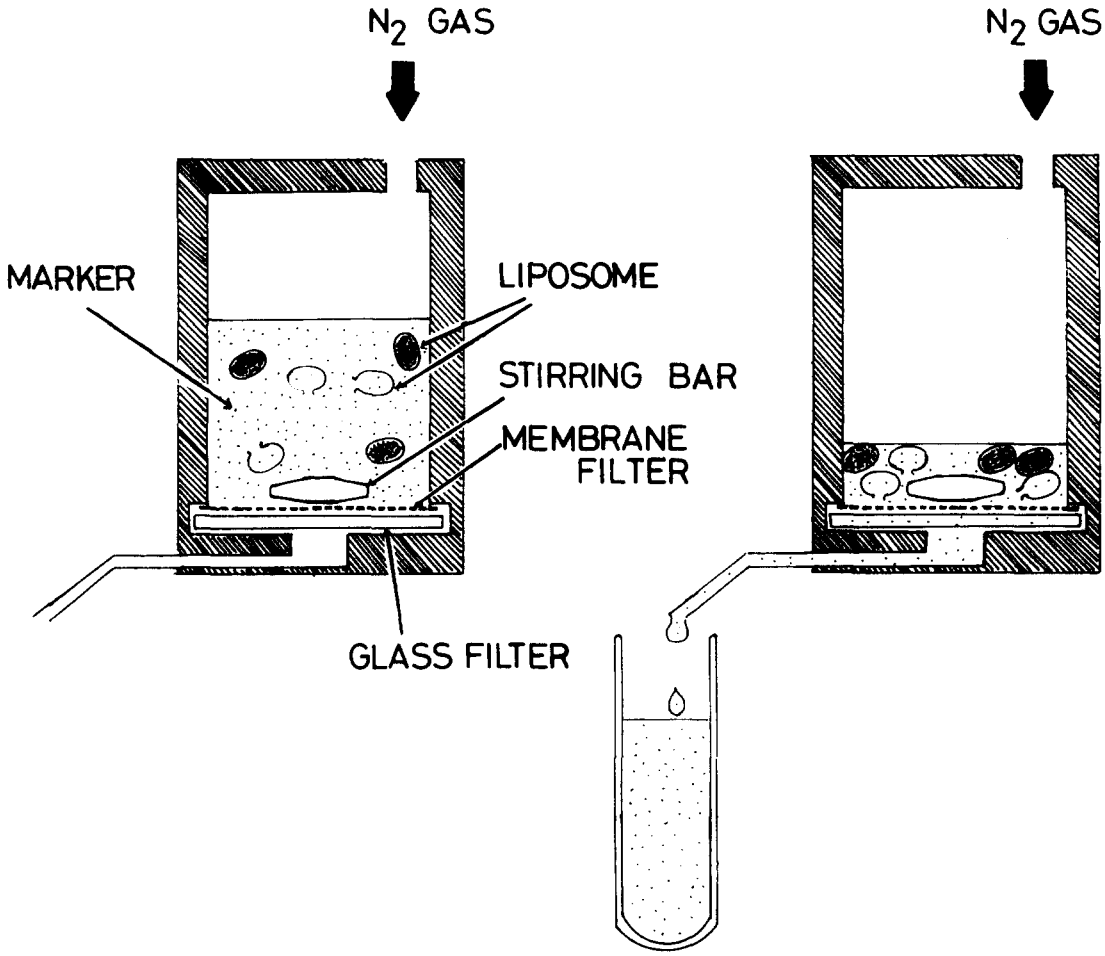

Fig. 1. Cell used for measurements of liposomal permeability. Liposome suspensions (2 ml) in the ultrafiltration cells were filtered through the membrane filter under pressure from N_2 gas. The first 1 ml of filtrate was collected. Radioactivity in an aliquot (0.5 ml) of the filtrate was counted. For details see text.

Results

Validity of the filtration method

Using the procedure described in Materials and Methods and liposome preparations containing [^{14}C]cholesterol as a marker of liposomal membranes and [^{3}H]glucose as a trapped marker, we first examined the effectiveness of various membrane filters. The amount of the untrapped glucose in the preparation was measured enzymically. The recoveries (percentage) of [^{14}C]cholesterol and [^{3}H]glucose in the filtrates are shown in Table I. The value of [^{3}H]glucose was corrected by subtracting the amount of untrapped glucose. Judging from the recovery of cholesterol in the filtrates obtained by this procedure ('Amicon' procedure), all the membranes tested provide an adequate barrier

TABLE I

SELECTION OF MEMBRANE FILTER

(A) Filtration of liposome preparations composed of egg yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 10/1/5). Liposomes composed of egg yolk phosphatidylcholine, dicetyl phosphate, cholesterol (molar ratio, 10/1/5) and a trace of [^{14}C]cholesterol were swollen in veronal-buffered saline containing a trace of [^{3}H]glucose. Untrapped glucose was removed by dialysis for more than 2 h against 0.15 M NaCl. Aliquots (2 ml) of the liposome preparation were filtered through membrane filters of various pore sizes. Filtration was induced by N_2 gas pressure ('Amicon cell' method) or by aspiration ('aspiration' method). In the 'Amicon cell' method, the radioactivity in an aliquot (0.5 ml) of the first 1 ml of filtrate was counted, while in the 'aspiration' method, the radioactivity in 0.5 ml of the total filtrate was counted. The recovery of markers from the filtrates was calculated from the corrected values (four times measured radioactivity, in both systems). (B) Filtration of liposome preparations composed of dipalmitoylglycerophosphocholine and dicetyl phosphate (molar ratio, 10/1. The procedure was as for (A), but diapalmitoylglycerophosphocholine was used instead of egg yolk phosphatidylcholine. Data are the percent recovery of markers from filtrate. Glc, glucose; Chol, cholesterol.

Procedure employed for filtration:		Type of liposomes							
		Multilamellar				Sonicated			
		Amicon cell		Aspiration		Amicon cell		Aspiration	
Markers:		Glc	Chol	Glc	Chol	Glc	Chol	Glc	Chol
Filter used									
Type	Pore size (μM)								
(A)									
GS	0.22	5	0	62	0	0	0	0	0
EG	0.2	16	15	53	35	52	26	59	67
PH	0.3	5	0	39	0	0	0	0	0
HA	0.45	16	0	54	0	10	0	13	4
DA	0.65	24	0	65	0	40	0	45	21
RA	1.2	28	0	51	21	34	0	60	46
(B)									
GS	0.22	0	0	5	0	0	0	8	0
EG	0.2	0	2	14	1	28	7	36	15
PH	0.3	0	0	12	0	0	0	5	0
HA	0.45	0	0	3	0	0	0	4	0

against multilamellar liposomes except type EG membranes: 15% of the cholesterol passed through EG membranes, which are made of cellulose acetate, indicating that some of the smaller liposomes or fragments of liposomes passed through these membranes. Even with membranes of 1.2 μm pore size, no appreciable cholesterol was detected in the filtrates. With membranes of over 0.45 μm pore size, appreciable amounts of glucose were detected in the filtrates (16—28%), indicating that the liposomes were damaged during filtration. When filtration was induced by aspiration ('aspiration' procedure), multilamellar liposomes again did not pass through any of the membranes except type EG and RA membranes, but there was appreciable loss of glucose under conditions when no cholesterol was recovered in the filtrates. Thus the 'aspiration' procedure caused greater damage to liposomes than the 'Amicon' procedure, possibly because the liposomes were forced to be squeezed on the surface of the membranes or in the membranes. Unexpectedly, the 'Amicon' procedure was found

to be applicable to sonicated liposomes, judging from the fact that no cholesterol was detected in filtrates except with type EG membranes. Type GS, PH, HA, DA and PA membranes are composed of mixtures of cellulose acetate and cellulose nitrate, while type EG membranes consist of cellulose acetate only. The effectiveness of these membranes may depend on the materials from which they are made. The finding that sonicated liposomes, which should be smaller (diameter, about 50 nm) than the average pore size of membrane filters, are retained on the membranes indicates that liposomes interact with the membrane filters, probably hydrophobic ally, and become fixed on the surface or in the matrix of the membranes.

As shown in Table IB, results on dipalmitoylglycerophosphocholine liposomes were similar to those on egg yolk phosphatidylcholine liposomes. In subsequent experiments, type PH membranes (pore size, 0.3 μm) was used, because these membranes resulted in least liposomal damage.

Effects of polyene antibiotics and digitonin on liposomes

Multilamellar liposomes composed of egg yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 10/1/5) were prepared in 0.3 M glucose, and incubated with various amounts of amphotericin B for 30 min at room temperature. Release of [^{3}H]glucose and [^{14}C]dextran from these liposomes is shown in Fig. 2. The percentage release of dextran was proportional to the percentage loss of glucose, and both were dependent on the concentration of antibiotic. It is significant, however, that the percentage release of dextran was lower than that of glucose. When this experiment was repeated using liposomes prepared in veronal-buffered saline, no significant loss of either glucose or dextran was detected (Table II).

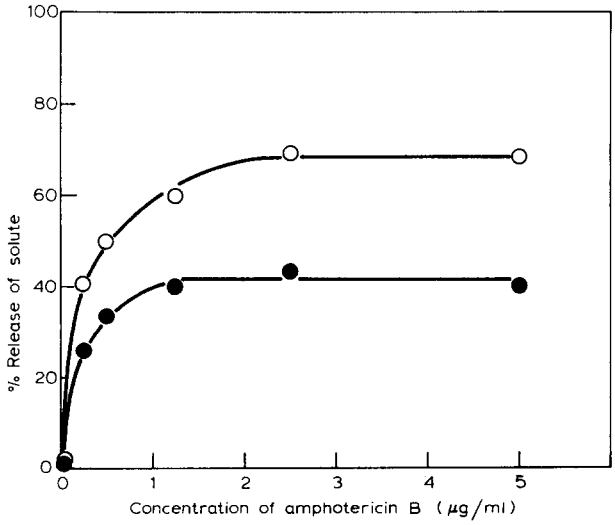

Fig. 2. Effect of amphotericin B on liposomes composed of egg yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 10/1/5). Liposomes containing traces of [^{3}H]glucose and [^{14}C]-dextran in their aqueous compartments were incubated with various amounts of amphotericin B for 30 min at room temperature. The releases of glucose (○) and dextran (●) were determined.

TABLE II

RELEASE OF GLUCOSE AND DEXTRAN FROM LIPOSOMES BY POLYENE ANTIBIOTICS AND DIGITONIN

Multilamellar liposomes (swollen in a solution of 0.3 M glucose containing traces of [^{3}H]glucose and [^{14}C]dextran or in a solution of veronal-buffered saline containing the same radioactive markers) were prepared from mixtures of egg yolk phosphatidylcholine, dicetyl phosphate and cholesterol in a molar ratio of 10/1/5. Liposomes were incubated with 2.0 μg/ml of amphotericin B, 20 μg/ml of nystatin, 20 μg/ml of filipin and 20 μg/ml of digitonin for 30 min at room temperature. Release of markers was calculated from the counts recovered from filtrates as described in text. Data are percent marker released.

	Liposomes swollen in			
	0.3 M glucose		Veronal-buffered saline	
Marker: Liposomes treated with	[^{3}H]Glucose	[^{14}C]Dextran	[^{3}H]Glucose	[^{14}C]Dextran
Amphotericin B	65.8	37.2	3.9	0.4
Nystatin	63.6	34.2	4.0	5.7
Filipin	73.2	59.5	58.4	62.2
Digitonin	100	100	100	—

Nystatin had a similar effect to amphotericin B. The effect of the glucose concentration in the aqueous compartments of liposomes on their sensitivity to nystatin was next examined (Fig. 3). With decrease of glucose concentration, the sensitivity of liposomes to nystatin decreased, and judging from glucose release, liposomes containing less than 100 mM glucose were almost

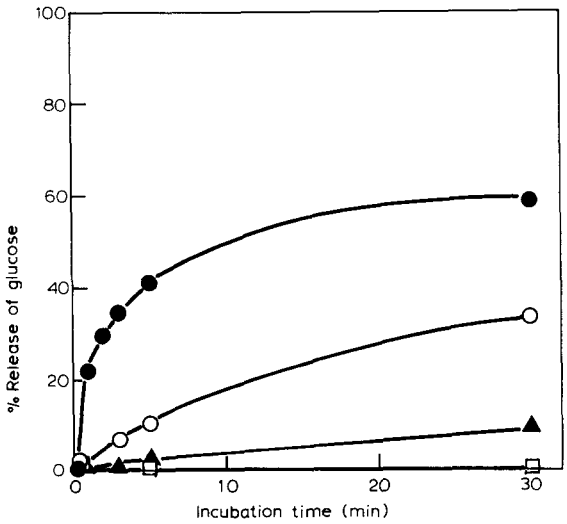

Fig. 3. Effect of nystatin on egg yolk phosphatidylcholine liposomes swollen in aqueous solution containing various concentrations of glucose. Liposomes composed of egg yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 10/1/5) were swollen in solution of 300 mM (300 mosM) glucose (●), 200 mM (200 mosM) glucose and 50 mM (100 mosM) NaCl (○), 100 mM (100 mosM) glucose and 100 mM (200 mosM) NaCl (▲) or 50 mM (50 mosM) glucose and 125 mM (250 mosM) NaCl (□). Untrapped glucose was removed by dialysis against physiological saline. Aliquots (40 nmol phosphatidylcholine) of the liposome suspension were treated with 20 μg/ml of nystatin at room temperature for various periods. Glucose release was assayed enzymically as described in the text.

entirely insensitive to the antibiotic. The glucose concentration in the aqueous compartments had a similar effect on the sensitivity of liposomes to amphotericin B (data not shown). Unlike these two polyene antibiotics, filipin caused release of both markers, even from liposomes prepared in veronal-buffered saline (Table II). Digitonin also caused release of markers from liposomes prepared in 0.3 M glucose as well as from those prepared in veronal-buffered saline.

Effect of lipid phase transitions on the permeability of liposomes

Near the gel to liquid crystalline phase transition temperature, the barrier properties of liposomes change profoundly, resulting in enhanced loss of trapped solutes [5,7—10]. Perturbation of the phase equilibrium by temperature variation resulted in marked increase in glucose loss through a dipalmitoylglycerophosphocholine bilayer [5,9]. Liposomes composed of dipalmitoylglycerophosphocholine and dicetyl phosphate, which have a transition temperature of 41°C determined by differential scanning calorimetry, were incubated at 47°C for various periods (Fig. 4). For this, suspensions of liposomes were first kept at room temperature (20°C) and then the temperature

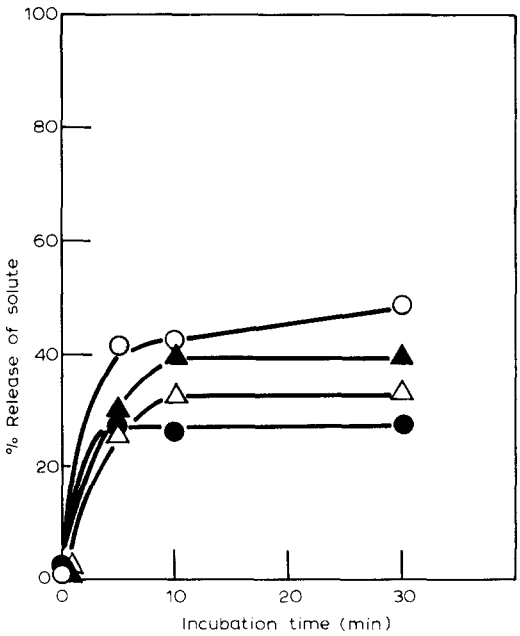

Fig. 4. Time course of marker release from dipalmitoylglycerophosphocholine liposomes on incubation at about the phase transition temperature. Liposomes composed of dipalmitoylglycerophosphocholine and dicetyl phosphate (molar ratio, 10/1), were stood at 20°C and then incubated for various periods at 47°C. Leakage of markers from the liposomes was assayed immediately after shifting the temperature back to 20°C by immersing the tubes in cold water. Liposomes were swollen in solutions of [^{3}H]glucose and [^{14}C]sucrose, [^{3}H]glucose and [^{14}C]dextran, [^{14}C]sucrose and [^{3}H]inulin, and [^{3}H]inulin and [^{14}C]dextran. Releases of each marker were calculated from average values for two sets of experiments using liposomes swollen in aqueous solution containing two kinds of markers. Release of glucose (○), sucrose (▲), inulin (△) and dextran (●) from liposomes.

of the test tube containing the suspension was raised to 47°C for various periods. Measurement of release of markers from the liposomes showed that the release of all markers tested was biphasic. Most of the markers were released during the first 5 min period of incubation (5 min). The temperature dependence of the change in permeability is shown in Fig. 4. Liposomes prepared in 0.3 M glucose and those prepared in veronal-buffered saline were incubated at various temperatures for 30 min. As shown in Fig. 5A, enhanced release of all trapped solutes from liposomes was demonstrated in the vicinity of the transition temperature. Though the extent of the increase of release of dextran was different from that of glucose, dextran, a macromolecular marker, could apparently be released from liposomes that had been prepared in 0.3 M glucose. Replacement of 0.3 M glucose by veronal-buffered saline did not affect glucose release (Fig. 5B) but suppressed sucrose release significantly. No appreciable release of dextran from liposomes was observed, even at the highest temperature tested (50°C).

Permeability properties of egg yolk phosphatidylcholine liposomes

Egg yolk phosphatidylcholine liposomes prepared in 0.3 M glucose were incubated at 50°C and 0°C for various periods (Fig. 6). These respective temperatures were chosen as being above and close to the phase transition temperature of egg yolk phosphatidylcholine (−10°C—0°C). At 50°C, only

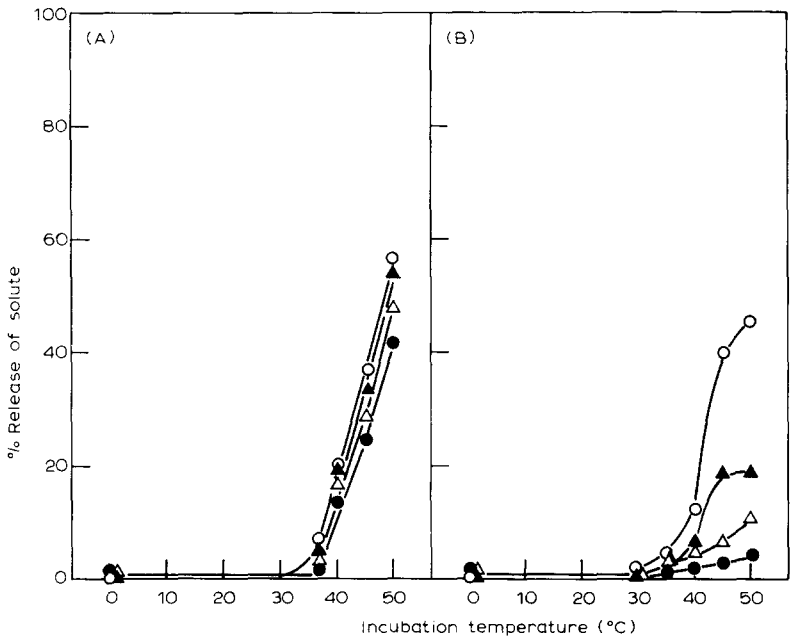

Fig. 5. Temperature dependence of release of various markers from dipalmitoylglycerophosphocholine liposomes. Liposomes composed of dipalmitoylglycerophosphocholine and dicetyl phosphate (molar ratio, 10/1) were incubated at various temperatures, and brought back to 20°C for assay of marker release. (A) Results obtained with liposomes swollen in 0.3 M glucose containing [^{3}H]glucose (○), [^{14}C]-sucrose (▲), [^{3}H]inulin (△) or [^{14}C]dextran (●). (B) Results with liposomes swollen in veronal-buffered saline containing the same markers as those used in (A).

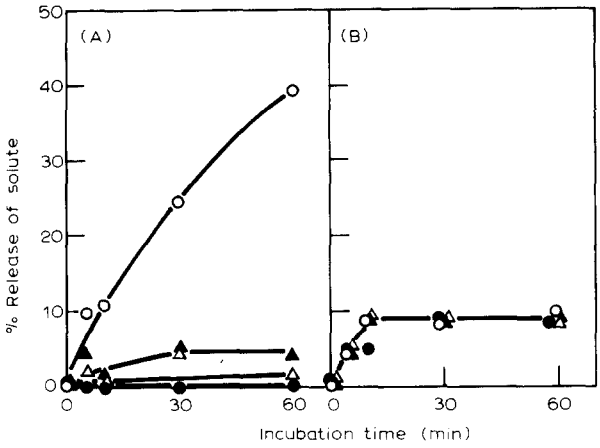

Fig. 6. Release of markers from egg yolk phosphatidylcholine liposomes on incubation at 50°C and 0°C. Liposomes composed of egg yolk phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio, 10/1/5) were swollen in 0.3 M glucose containing [^{3}H]glucose (○), [^{14}C]sucrose (▲), [^{3}H]inulin (△) or [^{14}C]dextran (●), and incubated at 50°C (A) or 0°C (B) for various periods.

glucose can permeate the liposomes significantly while at 0°C, all the markers permeated the liposomes equally well. The leak of glucose at 50°C was rather gradual, while that of markers including glucose at 0°C occurred during the initial period of incubation. This permeability change of egg yolk phosphatidylcholine liposomes observed at 0°C is similar to that of dipalmitoylglycerophosphocholine liposomes near the phase transition temperature. Similar results were also obtained with liposomes prepared without cholesterol. The temperature dependence of the increase in permeability at lower and higher temperatures is shown in Fig. 7. For these results, the liposomes were incubated for 1 h at various temperatures and then the temperature was raised or lowered to 20°C again and the suspension was filtered at 20°C. The permeability

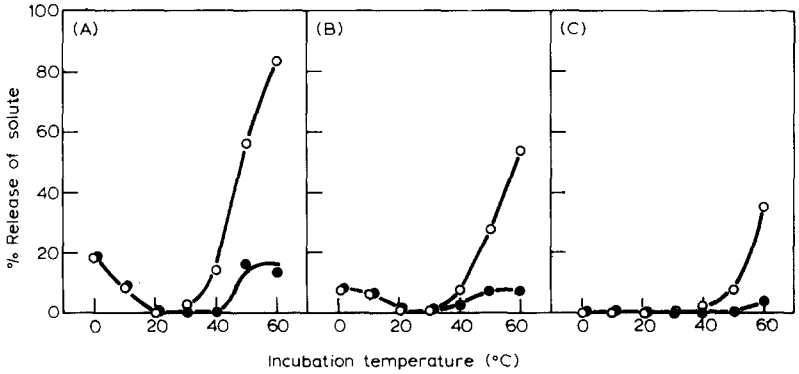

Fig. 7. Temperature dependence of release of glucose and sucrose from egg yolk phosphatidylcholine liposomes. Liposomes composed of egg yolk phosphatidylcholine and dicetyl phosphate (molar ratio, 10/1) with cholesterol in a molar ratio of 0 (A), 0.5 (B) or 1.0 (C) to phosphatidylcholine, were incubated for 1 h at various temperatures. The releases of [^{3}H]glucose (○) and [^{14}C]sucrose (●), trapped together in the same liposomes, were assayed.

change observed in both temperature ranges decreased with increase in the cholesterol content of the liposomes.

Discussion

In this work, using Amicon cells equipped with a membrane filter, we examined the rates of leakage of various markers, such as glucose, sucrose, inulin and dextran, from liposomes. The method employed, using filtration under pressure from N_2 gas, is better than the 'aspiration' method, in which filtration is induced by aspiration, since the aspiration method damages the liposomes by squeezing them on the membrane surface. In the present procedure damage of liposomes on the membrane was avoided by continuous gentle stirring of the liposome solution and by filtration of only about half the solution in the filtration cell. Choice of a suitable filter was also found to be important. With a suitable filter the permeability of liposome membranes to various markers could be measured rapidly and accurately.

It is known that perturbation of lipid bilayers by various treatments results in change in permeability. It is known that the increase of permeability induced by ionophores (see reviews of Kinsky [11] and Gomperts [12]) and polyene antibiotics [11,13] depends on the properties of the permeating compound. Phospholipase C, saponin and activated complements were found to cause nonspecific increase in permeability [14].

Amphotericin B and nystatin are known to be polyene antibiotics that form aqueous pores of about 8 Å diameter in lipid bilayers [13]. Thus, these antibiotics produced rather specific permeability changes. De Kruijff et al. [13] reported that amphotericin B and nystatin produced a specific permeability change; that is, rapid leak of K^+ with slow (amphotericin B) or no (nystatin) efflux of glucose. In the present experiments, both amphotericin B and nystatin induced change in permeability to not only glucose but also dextran, when the liposomes contained 0.3 M glucose in their aqueous compartments. This result appears to contradict the observation of de Kruijff et al. [13], who used liposomes containing equal amounts of KCl and glucose. This prompted us to examine the sensitivities to nystatin of liposomes swollen in aqueous solutions containing various concentrations of glucose (Fig. 3). We found that on treatment with nystatin no appreciable glucose was released from liposomes containing 100 mM glucose and 100 mM NaCl in their aqueous compartments, as observed by de Kruijff et al. [13]. We also obtained similar results with amphotericin B. According to de Kruijff et al [13], the pores produced by amphotericin B must have a diameter similar to the hydrodynamic radius of glucose, since glucose permeated slowly through liposomes treated with this antibiotic. We found that amphotericin B did not change the permeability to glucose, unless the liposomes contained more than 200 mM glucose in their aqueous compartments. It seems likely from these findings that amphotericin B and nystatin first form pores of about the same size in the liposomal membranes, and that, only Na^+ and Cl^- can penetrate through these pores. These findings are in good agreements with those of de Kruijff et al. [13] and also with results on black lipid membranes [15,16]. The probable explanation of why liposomes seem to be secondarily damaged by the antibiotics when

they contained more than 200 mM glucose, is that on treatment of liposomes with amphotericin B or nystatin, Na^+ and Cl^- enter the liposomes freely. Then as a result of the low permeability to trapped glucose (efflux), the liposomes become swollen with severe damage to their bilayers. As a results not only glucose but also the macromolecular marker dextran (M_r 75 000) is released from the liposomes.

It is now generally accepted that lysis of living cells by various reagents proceeds by a colloid-osmotic mechanism due to the formation of membrane lesions that are initially too small to permit the escape of large charged molecules such as hemoglobin, but that are sufficiently large to allow the exchange of cations. This process is presumably also responsible for the leakage of macromolecules from liposomes, but, in the present work these large charged molecules (proteins) were replaced by glucose. As pointed out by Inoue and Kinsky [17] and Kinsky [18], important differences between liposomes and cells such as erythrocytes are that cells are single aqueous compartments, bounded by a membrane, which contain a high concentration of proteins, and are sensitive to a colloid-osmotic mechanism, whereas liposomes consist of lipid bilayers separating aqueous regions that contain only trapped low molecular weight markers, and they are generally insensitive to a colloid-osmotic mechanism. In fact, no information has so far been obtained about how a 'colloid-osmotic mechanism' might contribute to the process of liposomal damage induced by agents attacking the membrane, although it is well known that multilamellar liposomes behave as ideal osmometers when there is an osmotic gradient between their inside and outside. The capacity of liposomes to swell before they burst and heal must be limited. This swelling might, of course, be accompanied by a loss of solutes from the liposomes. The present observations clearly indicate that a 'colloid-osmotic mechanism' brings about the rupture of liposomes, probably with temporary formation of structures that have lost the form of closed vesicles. This conclusion suggests the need for caution in interpreting results on increased liposomal permeability under the influence of various reagents, especially when the liposomes contain various solutes in their aqueous compartments. Furthermore, it is noteworthy that with non-sonicated liposomes composed of egg yolk phosphatidylcholine, dicetyl phosphate and cholesterol, 200 mosM of 'impermeable' substances in the aqueous compartments rendered the liposomes sensitive to a 'colloid-osmotic' mechanism, whereas less than 100 mosM of 'impermeable' solutes did not induce sufficient swelling, which eventually cause the rupture of the liposome structure.

On treatment with filipin or digitonin, glucose loss was accompanied by release of macromolecules under conditions where the osmotic mechanism did not work. As proposed by de Kruijf et al. [13], it seems likely that filipin disrupted the liposomal membrane, so that both small molecules, such as glucose, and large molecules were released. Digitonin, which also attacks cholesterol in the membrane [19], had almost the same action as filipin on liposomal membranes. Consistent with the above proposal for the action of digitonin, rod-like structures could be seen by electron microscopy in preparations of liposomes treated with digitonin.

Perturbation of the lipid phase equilibrium by temperature change resulted in rather nonspecific increase in the release of trapped markers from liposomes

prepared in 0.3 M glucose. However, with liposomes, containing veronal-buffered saline instead of 0.3 M glucose, rather specific release of glucose was observed, indicating that the increased permeability of liposomes swollen in saline cannot be ascribed to 'macroscopic' rupture of the liposomal membranes. The preferential permeability to glucose suggests a size distribution and probability of statistical pore formation at the boundaries of liquid and solid domains: Most of the pores were probably not large enough to allow the escape of larger molecules, such as inulin and dextran. The fact that an osmotic mechanism was involved in the process also indicates that small ions such as Na^+ might be more permeable than glucose through the statistical 'pores'. In this connection it is interesting to cite a report [10] that in the vicinity of the phase transition temperature the increased permeability of liposomes decreases with increase in diameter of the permeating cations. The present investigation also suggests that the permeation through the 'pores' of cations such as Na^+ increased much more than that of glucose. With liposomes of dipalmitoyl-glycerophosphoglycerol, it was found that the rate of self-diffusion of $^{22}Na^+$ at the transition temperature increased much more than that of [^{14}C]sucrose [7]. Our data support this observation.

At a temperature well above the transition temperature, egg yolk phosphatidylcholine liposomes exhibited rather specific glucose release even when they had been swollen in 0.3 M glucose. This process did not abruptly induce an osmotic gradient, indicating that the permeation through bilayers took place mainly by individual diffusion of the permeants. Thus well above the phase transition temperature the permeation mechanism may be completely different from that observed during perturbation of phase equilibrium.

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