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EFFECTS OF TEMPERATURE AND CHOLESTEROL ON THE GLUCOSE PERMEABILITY OF LIPOSOMES PREPARED WITH NATURAL AND SYNTHETIC LECITHINS

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SUMMARY

Incorporation of cholesterol into liposomes can, under appropriate conditions, decrease the amount of trapped glucose which is released upon subsequent incubation of the liposomes at various temperatures. The ability of cholesterol to reduce the glucose permeability of liposomes is markedly influenced by the fatty acid substituents present in the lecithin used in preparation of the liposomes. The sterol had the greatest effect when liposomes were prepared from synthetic or natural lecithins (*e.g.*, (dioleoyl)-, (1-stearoyl-2-oleoyl)-, or egg lecithin) whose molecular area, as determined by monolayer studies, is significantly reduced by cholesterol. In contrast, cholesterol had a smaller effect on the release of glucose from liposomes prepared with (dilinoleoyl)-lecithin, particularly when the liposomes contained a low content of charged amphipathic compounds (dicetyl phosphate or phosphatidic acid); the molecular area of this lecithin is not diminished in the presence of sterol. Ergosterol and cetyl alcohol can also reduce the glucose permeability of egg lecithin liposomes whereas cholesterol esters have no influence. The significance of these observations, as regards the function of sterols in natural cell membranes, is discussed.

INTRODUCTION

The role of sterols in natural membranes has attracted much attention. Not only are sterols often found in high concentration in a variety of membrane systems but certain clinically effective drugs, such as the polyene antibiotics, induce permeability alterations and promote lysis as a consequence of interaction with sterols localized in the cell membranes of sensitive organisms[1]. Investigations with lipid monomolecular layers, oriented at the air–water interface, have demonstrated that cholesterol, for example, can reduce the mean molecular area of natural lecithins and certain synthetic phospholipids which contain specific fatty acid substituents[2]. These observations support the suggestion, frequently made, that a role of sterols may be to stabilize the arrangement of phospholipids in cell membranes.

The applicability of reactions occurring at an air–water interface to phenomena

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associated with natural cell membranes is, of course, open to question. Recently, BANGHAM, STANDISH AND WATKINS[3] demonstrated that aqueous lipid dispersions, in which the lipids were oriented at a water–water interface, offered an attractive alternative to monolayers as an experimental model membrane. Extensive X-ray and electron microscopic studies have shown that, in these lamellar dispersions (hereafter referred to as liposomes), the lipids are arranged in an array of concentric bilayers. BANGHAM, STANDISH AND WATKINS[3] demonstrated that liposomes could be prepared with different marker compounds trapped in the aqueous regions which separate the bilayers from each other and that these markers could be subsequently released by treatment of the liposomes with numerous surface-active and lytic agents.

We have previously shown that incorporation of cholesterol into liposomes prepared with egg lecithin reduced the amount of phosphate or glucose that was released when the liposomes were incubated at various temperatures[4]. The present investigation is a continuation of these experiments with particular emphasis on the influence of the phospholipid fatty acid substituents on the ability of sterols to reduce the permeability of the liposomes. For this purpose, extensive use was made of a simple and rapid spectrophotometric assay for following glucose release which is based on the formation of TPNH in the presence of hexokinase and glucose-6-phosphate dehydrogenase[5]. This assay requires much less liposomal material than other procedures which have been employed to study the leakage of trapped markers and, therefore, renders feasible the preparation of liposomes from some amphipathic lipids which are currently available in limited quantities. The results presented below indicate that less glucose was released when cholesterol or ergosterol was incorporated into liposomes, particularly when these were prepared with natural and synthetic lecithins whose molecular area is reduced by sterol.

MATERIALS AND METHODS

Chemicals and enzymes

The chemicals and enzymes were obtained from the following companies: Applied Science Laboratories, State College, Pa. (cholesterol acetate and cholesterol oleate); Boehringer-Mannheim Corporation, New York, N.Y. (hexokinase and glucose-6-phosphate dehydrogenase); K and K Laboratories, Inc., Plainview, N.Y. (dicetyl phosphate and cetyl alcohol); Pierce Chemical Company, Rockford, Ill. (egg lecithin); Rohm and Haas, Philadelphia, Pa. (Triton X-100); Sigma Chemical Company, St. Louis, Mo. (ATP, TPN, cholesterol, ergosterol, and Tris).

The synthetic lecithins were prepared by methods previously described[6]. Phosphatidic acid was derived from egg lecithin by treatment with phospholipase D; this material, as well as some of the synthetic lecithins, was kindly donated by Dr. J. DE GIER. The purity of all phospholipids was routinely checked by thin-layer chromatography on silica gel plates with chloroform–methanol–water (65:35:4, by vol.) and chloroform–methanol–6 M NH_4OH (70:30:5, by vol.) as solvent systems for the lecithins and phosphatidic acid, respectively. The purity of the sterols and sterol esters was determined by thin-layer chromatography with diethyl ether–hexane (70:30, v/v). A contaminant present in the ergosterol was removed by recrystallization of the sterol from ethanol.

Stock solutions of the phospholipids were prepared in chloroform and the

concentration of the lipids was determined by the procedure of GERLACH AND DEUTICKE[7] for total phosphate.

Preparation of liposomes

Liposomes were prepared by the "micro method" which has been described in detail in a previous paper[5]. In this procedure, 2 μmoles of lecithin and appropriate quantities of the other lipids (dicetyl phosphate, phosphatidic acid, sterols, or sterol esters), necessary to obtain the desired molar ratio, were added to 10-ml conical flasks. After removal of the chloroform with a stream of N_2 and subsequent evacuation, 0.2 ml of 0.3 M glucose was added to give a final lecithin concentration of 10 μmoles/ml. The dried lipid film, which had been deposited on the walls of the flask, was dispersed by agitation with a Vortex mixer under a N_2 atmosphere. The liposome preparation was kept at room temperature for 1.5 h, with occasional agitation, before dialysis to remove most of the untrapped glucose. Dialysis was performed for 1 h with gentle shaking against 150 ml of a solution containing 0.075 M KCl and 0.075 M NaCl (hereafter referred to as isotonic salt mixture). In some experiments, in which the liposomes were prepared from lecithins that were readily susceptible to oxidation (*e.g.*, (dilinoleoyl)lecithin), the dialyzing solution was first freed of dissolved O_2 by boiling, and then bubbling with N_2, and dialysis was carried out under an atmosphere of N_2.

Assay for leakage

The complete system contained, in cuvettes with a 10-mm light path, the following reagents (in order of addition): 0.28 ml of 0.1 M Tris buffer (pH 8); 0.50 ml of "double strength" isotonic salt mixture (0.15 M KCl and 0.15 M NaCl, prepared in Tris buffer); 0.10 ml of 0.02 M magnesium acetate; 0.05 ml of 0.02 M ATP; 0.05 ml of 0.01 M TPN^+; 5 μl of hexokinase (6.7 mg protein per ml); and 5 μl of glucose-6-phosphate dehydrogenase (3.4 mg protein per ml). Control cuvettes were identical

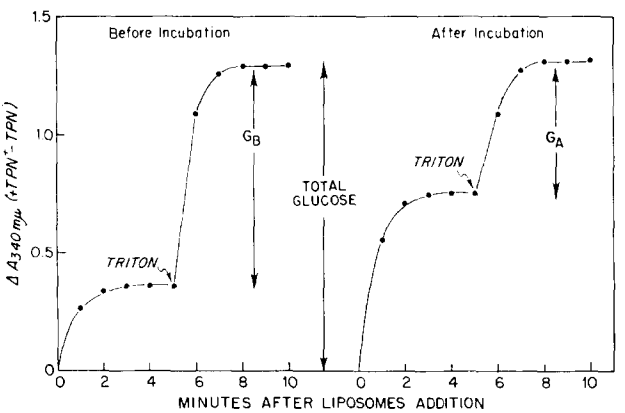

Fig. 1. Experiment to illustrate application of spectrophotometric assay. Liposomes (5 μl), prepared from egg lecithin and dicetyl phosphate in a molar ratio of 7:2, were added to experimental (+TPN^+) and control (−TPN^+) cuvettes at zero time. Triton (0.1 ml), to give a final concentration of 1%, was added to both cuvettes at 5 min. The values on the ordinate are the differences in absorbance (corrected for dilution following each addition) between the experimental and control cuvettes. The curve on the left was obtained with liposomes assayed immediately after dialysis; that on the right was obtained after the liposomes had been incubated 1 h at 41°. For additional details, see text and ref. 5.

to the above except that TPN^+ was omitted. The reaction was started by the addition of 5 μl of the appropriate liposome preparation.

Experiments which establish the validity of the spectrophotometric assay and demonstrate its application to a study of the kinetics of glucose release induced by lytic agents, such as filipin, have been published[5]. The basis for this assay, and its use in the present investigation, is illustrated in Fig. 1. As shown previously[5], any untrapped glucose in the liposome preparation can be measured by the difference in absorbance at 340 mμ between the experimental and control cuvettes before the addition of a lytic agent. The total glucose can be determined from the difference in absorbance of the cuvettes after addition of 0.1 ml of a 10% Triton solution. Under the above conditions, Triton has been shown to induce complete lysis of the liposomes, and liberation of all the trapped marker, within 2 min (ref. 5).

In the experiments described below, the effect of temperature on the amount of glucose released was determined by the following procedure. Immediately after dialysis, the liposomes were assayed for the amount of trapped marker (equivalent to the total *minus* the untrapped glucose) present in the preparation. Appropriate aliquots of the liposomes (30–150 μl) were then transferred to small test tubes (5 mm × 38 mm) which were flushed with N_2 and sealed with plastic caps to prevent loss of water by evaporation. After incubation for various times and at different temperatures, as indicated, the liposomes were reassayed to determine the decrease in the amount of trapped marker. It is apparent from the experiment described in Fig. 1 that this decrease is reflected by a corresponding increase in the level of untrapped glucose since the total amount of marker present in the preparation must remain constant. Controls were always included in every assay to verify that the total glucose present before and after incubation was the same; the few experiments in which these values did not agree within 5% of each other were discarded. The percent of glucose which remained trapped can, therefore, be calculated from the expression: 100 (G_A/G_B), where G_A is the amount of glucose released by Triton after incubation of the liposomes and G_B is the amount of glucose released by Triton before incubation of the liposomes. In the following section, the results (average of 2 or more experiments) are given as the percent of trapped marker, initially contained in the liposome preparation, which was released under the conditions of incubation. This was calculated from the equation:

$$\text{"Percent maximum glucose released"} = 100 - 100\ (G_A/G_B).$$

RESULTS

Effect of incubation time and liposome concentration

The experiments described below are concerned with the effect of liposome composition on the amount of glucose released at various temperatures after incubation for 1 h. This time interval was chosen because, as seen in Fig. 2, the amount of glucose released within 1 h provides an approximate measure of the rate at which the marker is lost. Fig. 3 demonstrates that the rate of glucose release was not influenced by liposome concentration. This apparent "zero-order" dependence on liposome concentration indicates that it was unnecessary to compensate for the amount of isotonic salt mixture which had entered the dialysis sac during the preparation of the liposomes.

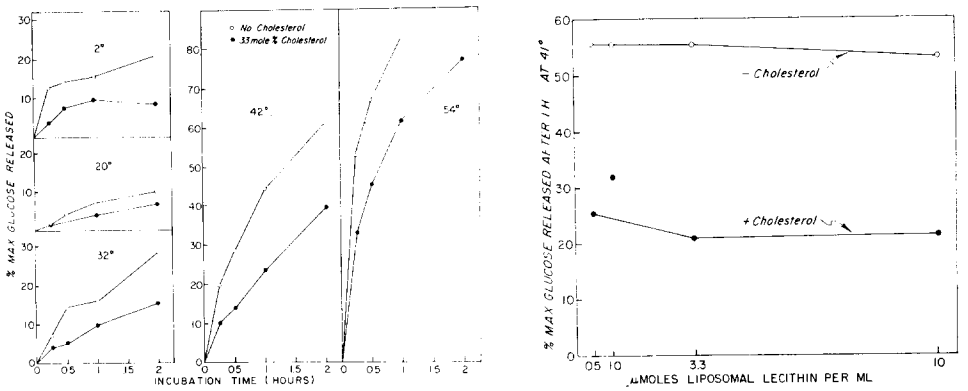

Fig. 2. Time course of glucose release at various temperatures. Liposome composition (molar ratios): egg lecithin, 7; dicetyl phosphate, 2 (○) and egg lecithin, 7; dicetyl phosphate, 2; cholesterol, 3.5 (●).

Fig. 3. Effect of liposome concentration on the percent of trapped glucose released after incubation for 1 h at 41°. Liposome composition (molar ratios): egg lecithin, 7; dicetyl phosphate, 2 (○) and egg lecithin, 7; dicetyl phosphate, 2; cholesterol, 3.5 (●). The amount of glucose released was determined by the standard procedure except that each liposome preparation was diluted with isotonic salt mixture, to give the lecithin concentrations indicated on the abscissa, before assay.

Effect of sterols

Figs. 2 and 3 also illustrate that significantly less glucose was released from liposomes prepared with egg lecithin when cholesterol was incorporated. These results thus confirm our earlier experiments[4] which employed a different method for following the leakage of trapped markers. The effect of different cholesterol concentrations is shown in Figs. 4 and 5. Although there is no evidence to indicate that all of the sterol had actually been incorporated into the liposomal bilayer structure, it is apparent

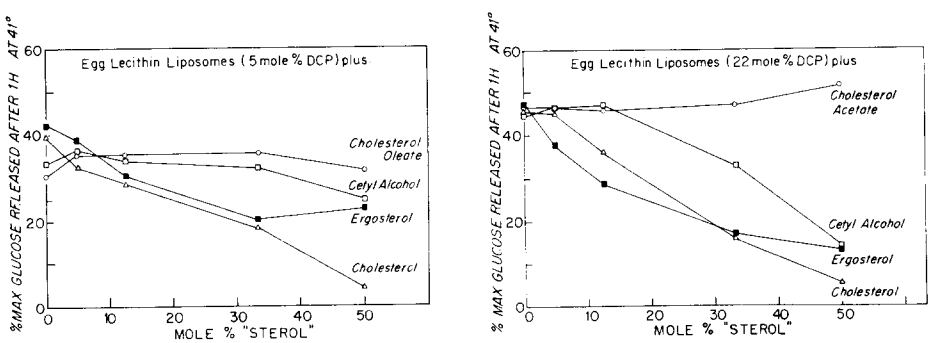

Fig. 4. Effect of sterol incorporation into egg lecithin liposomes on the relative amount of glucose released after 1 h at 41°. Liposomes were prepared from egg lecithin and dicetyl phosphate (DCP) in a molar ratio of 7:0.35, respectively (5 mole % dicetyl phosphate), with varying amounts of the indicated "sterol". In this, and subsequent, experiments the content of dicetyl phosphate was not included in the calculation of the "mole % sterol" which is given by the expression: 100 × (moles sterol/moles sterol + moles lecithin).

Fig. 5. Effect of sterol incorporation into egg lecithin liposomes on the relative amount of glucose released after 1 h at 41°. Experiment similar to that described in the legend to Fig. 4 except that the liposomes contained egg lecithin and dicetyl phosphate (DCP) in a molar ratio of 7:2, respectively (22 mole % dicetyl phosphate).

that increasing levels of cholesterol reduced the amount of glucose released after incubation for 1 h at 41°. For reasons indicated below, these experiments were performed with liposomes containing different amounts of dicetyl phosphate (5 and 22 mole %, respectively). Ergosterol and cetyl alcohol can substitute for cholesterol and decrease the loss of glucose from liposomes (particularly when the liposomes contain a relatively high amount of dicetyl phosphate) whereas cholesterol esters are completely ineffective*.

Influence of lecithin fatty acid substituents on the effect of sterols

As noted in INTRODUCTION, the argument that sterols may function in cell membranes to stabilize the bilayer configuration of phospholipids is based mainly on the observation that, in mixed monolayers of natural lecithins and cholesterol, the mean molecular area of the phospholipid was significantly reduced. The results of the preceding experiments with egg lecithin liposomes are consistent with this postulated role for sterols. However, monolayer studies performed with synthetic phospholipids have shown that cholesterol does not always produce a significant reduction in cross-sectional area and that the extent of area reduction is markedly influenced by the type of fatty acids present in the phospholipid[2].

Accordingly, the above experiments were repeated with liposomes prepared from several synthetic lecithins to determine if the degree of reduction of glucose permeability was similarly influenced by the nature of the fatty acid substituents. Figs. 6 and 7 indicate that increasing concentrations of the sterol result in a decreased

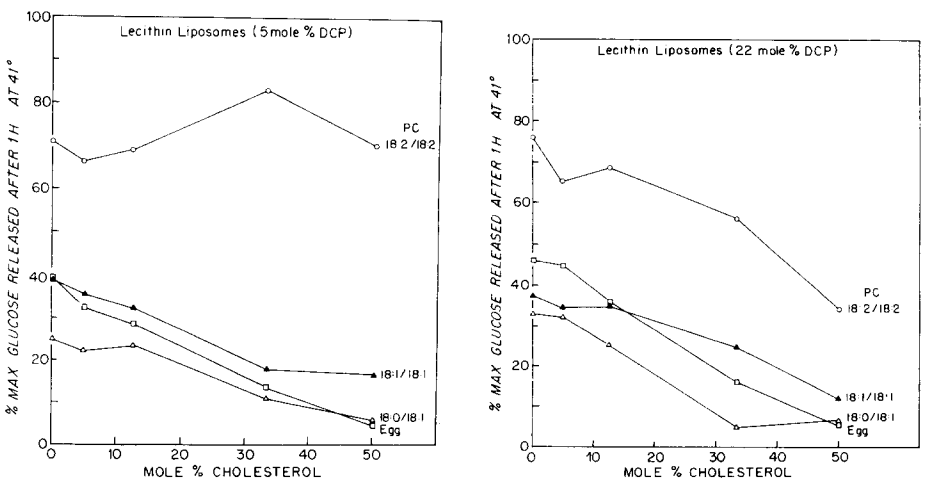

Fig. 6. Effect of cholesterol incorporation into liposomes prepared with natural and synthetic lecithins (PC) on the relative amount of glucose released after 1 h at 41°. Liposomes were prepared from the indicated phospholipid and dicetyl phosphate (DCP) in a molar ratio of 7:0.35, respectively (5 mole % dicetyl phosphate), with varying amounts of cholesterol.

Fig. 7. Effect of cholesterol incorporation into liposomes prepared with natural and synthetic lecithins (PC) on the relative amount of glucose released after 1 h at 41°. Experiment was similar to that described in the legend to Fig. 6 except that the liposomes contained the indicated phospholipid and dicetyl phosphate (DCP) in a molar ratio of 7:2, respectively (22 mole % dicetyl phosphate).

* Cholesterol esters and cetyl alcohol were examined because previous experiments have demonstrated that the polyene antibiotic, filipin, can interact with monolayers of cetyl alcohol, but not sterol esters, at low molar ratios of antibiotic to spread lipid[8].

loss of marker from liposomes prepared from (dioleoyl)- and (1-stearoyl-2-oleoyl)-lecithin. It should be emphasized that the cross-sectional area of these lecithins is markedly reduced by cholesterol[2]. In contrast, cholesterol had a much smaller effect on liposomes prepared from (dilinoleoyl)lecithin. Indeed, no effect of the sterol was observed with (dilinoleoyl)liposomes containing 5 mole % dicetyl phosphate. These results appear significant because cholesterol has no appreciable condensing effect on monolayers of this lecithin[2]. It should also be noted (as indicated in Figs. 6, 7, 8 and 12) that liposomes prepared from (dilinoleoyl)lecithin lose much more glucose at temperatures above 20° than liposomes made from either (dioleoyl)-, (1-stearoyl-2-oleoyl)- or egg lecithin. These findings are in agreement with monolayer studies which have demonstrated that the area per molecule increases with an increased number of unsaturated bonds in the paraffinnic chain. These observations further suggest that the glucose permeability of the liposomes may be dependent on the "closeness", *i.e.* packing, of phospholipids in the bilayer (see DISCUSSION).

Effect of temperature

The preceding experiments were performed at 41°. The effect of different temperatures on glucose release from various liposomes is shown in Fig. 8 and the ratio of marker released from liposomes prepared in the absence and presence of

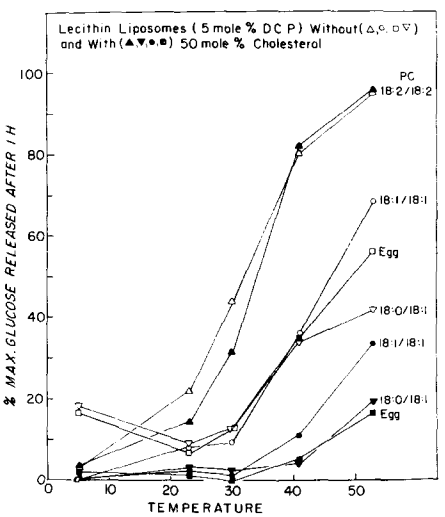

Fig. 8. Effect of temperature on the percent of trapped glucose released after incubation for 1 h from liposomes prepared with and without cholesterol. Liposomes were prepared from the indicated phospholipid (PC) and dicetyl phosphate (DCP) in a molar ratio of 7:0.35, respectively (5 mole % dicetyl phosphate), without (open symbols) and with (closed symbols) 50 mole % cholesterol.

50 mole % cholesterol is plotted in Figs. 9 and 10. It is apparent that cholesterol also has a significant reductive effect on glucose permeability at 53° (the highest temperature tested). The amount of marker released below 30° was generally too small to warrant calculation of the ratios at these temperatures. Again, it should be emphasized that, for the compounds tested, the effect of cholesterol is most pronounced with liposomes prepared from lecithins whose cross-sectional area is reduced by the sterol.

In previous experiments with egg lecithin liposomes it was observed that more marker (glucose or phosphate) was released at 2–5° than at 20–30° (ref. 4; see also Fig. 2). In the present investigation, no minima in the temperature *vs.* release curve was observed with liposomes prepared from either (dioleoyl)- or (dilinoleoyl)lecithins. Most natural lecithins are, however, not di-substituted and contain instead a saturated and unsaturated fatty acid at the 1 and 2 positions, respectively. Therefore, it is

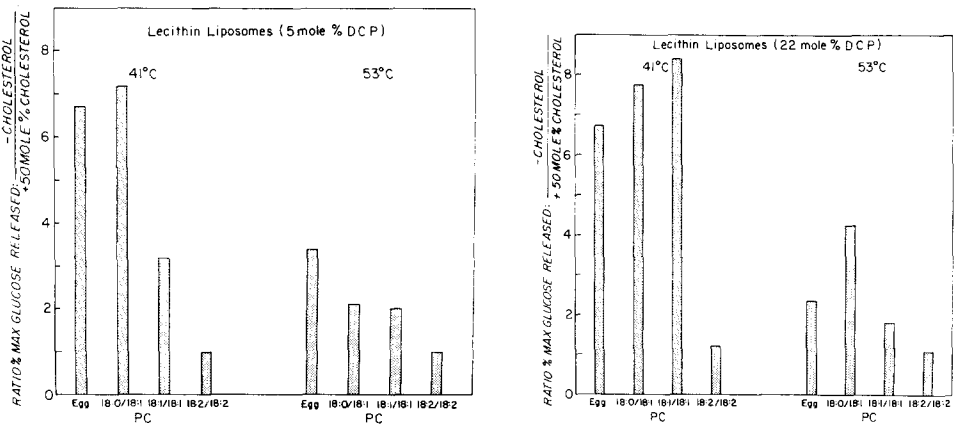

Fig. 9. Ratios of the percent of trapped glucose released from various liposomes after incubation for 1 h at 41° and 53°. Data taken from the experiment described in Fig. 8. DCP, dicetyl phosphate; PC, phosphatidyl choline.

Fig. 10. Ratios of the percent of trapped glucose released from various liposomes after incubation for 1 h at 41° and 53°. Experiment was similar to that described in the legend to Fig. 9 except that the liposomes were prepared from the indicated phospholipid (PC) and dicetyl phosphate (DCP) in a molar ratio of 7:2, respectively (22 mole% dicetyl phosphate), without and with 50 mole% cholesterol.

interesting to note that liposomes prepared with (1-stearoyl-2-oleoyl)lecithin also release more glucose at 2° than at 20° (Fig. 8).

Effect of phosphatidic acid

At the present time, it seems premature to attach any biological significance to the observation that liposomes, prepared with either egg or (1-stearoyl-2-oleoyl)-lecithin, lose less marker at "physiological" temperatures than in the "cold". When egg lecithin liposomes were made with phosphatidic acid (in place of dicetyl phosphate), the amount of glucose released at 2° was identical to the amount released at 30° (Fig. 11). It should be emphasized, however, that substitution of phosphatidic acid for dicetyl phosphate did not alter the effect of cholesterol. Thus, liposomes prepared from egg lecithin are still less permeable to glucose in the presence of the sterol, whereas cholesterol has no marked effect on liposomes prepared from (dilinoleoyl)lecithin (Fig. 12).

Effect of dicetyl phosphate

Increasing concentrations of dicetyl phosphate (5–22 mole %) had no influence *per se* on the amount of marker lost from egg lecithin liposomes (Fig. 13). Nevertheless, we have repeatedly observed an effect of high levels of dicetyl phosphate when in-

corporated into liposomes which also contain sterol or cetyl alcohol. For example, both ergosterol and cetyl alcohol caused a greater reduction in glucose permeability when egg lecithin liposomes were prepared with 22, instead of 5, mole % dicetyl phosphate (compare Figs. 4 and 5). Also, cholesterol had a definite influence on liposomes prepared from (dilinoleoyl)lecithin in the presence of 22 mole % dicetyl phosphate (Fig. 7), although the effect was still significantly less than that obtained with lipo-

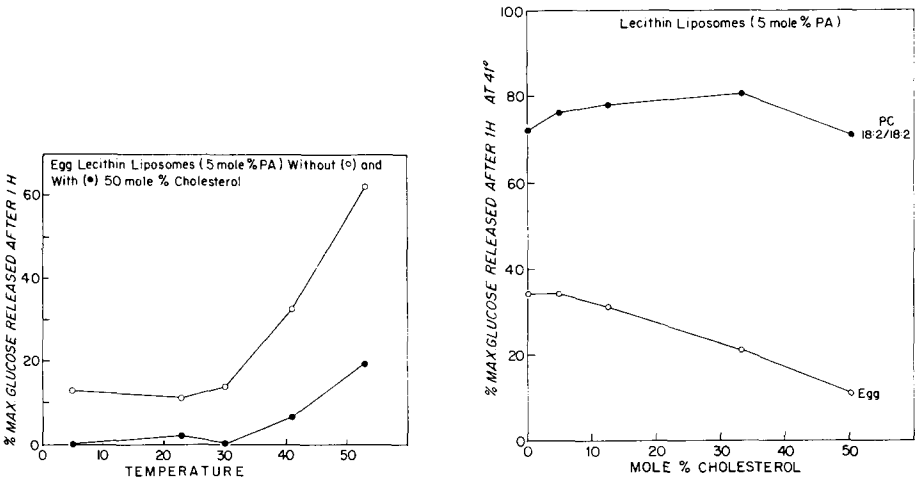

Fig. 11. Effect of temperature on the percent of trapped glucose released after incubation for 1 h from liposomes containing phosphatidic acid. Experiment was similar to that described in the legend to Fig. 8 except that the liposomes were prepared from egg lecithin and phosphatidic acid (PA) in a molar ratio of 7:0.35, respectively (5 mole % phosphatidic acid), without (open symbols) and with (closed symbols) 50 mole % cholesterol.

Fig. 12. Effect of cholesterol incorporation into liposomes prepared with natural and synthetic lecithins (PC), and phosphatidic acid (PA), on the relative amount of glucose released after 1 h at 41°. Liposomes were prepared with the indicated lecithin and phosphatidic acid in a molar ratio of 7:0.35, respectively (5 mole% phosphatidic acid), with varying amounts of cholesterol.

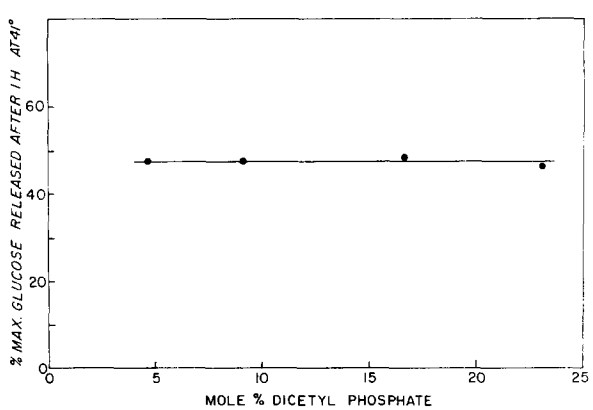

Fig. 13. Lack of an effect of dicetyl phosphate on the percent of trapped glucose released after 1 h at 41° from liposomes prepared with egg lecithin. Liposomes were prepared from mixtures containing varying molar ratios of egg lecithin and dicetyl phosphate to give the "mole % dicetyl phosphate" content indicated on the abscissa.

somes prepared from lecithins whose molecular area was reduced by the sterol (Fig. 10). Recent monolayer studies have shown that the molecular area of dicetyl phosphate is not affected by cholesterol[9] and further experiments are required to determine the basis for these effects of dicetyl phosphate.

DISCUSSION

The experiments described above have demonstrated that incorporation of cholesterol into liposomes can, under appropriate conditions, decrease the amount of glucose which is released upon subsequent incubation of the liposomes at various temperatures. Although we have studied the loss of trapped marker from liposomes, it should be emphasized that this effect of cholesterol appears to be a general phenomenon. In a parallel investigation, DE GIER, MANDERSLOOT AND VAN DEENEN[10] examined the penetration of glycerol and erythritol into liposomes at different temperatures and observed a similar reduction in permeability when the sterol was incorporated. Furthermore, both studies indicate that the effect of cholesterol was markedly influenced by the fatty acid substituents in the lecithin which was used for preparation of the liposomes. In this connection, it is also interesting to note that cholesterol can apparently lower the water permeability of thin lipid membranes prepared with egg lecithin[11].

The mechanism by which sterols reduce liposomal permeability must still be established but the available evidence suggests that ion-dipole interaction between lecithin and cholesterol probably does not occur to a significant extent (refs. 9, 12, 13; see, however, ref. 14). A more plausible alternative is that the sterol promotes a tighter packing of the phospholipid and that the stability of the bilayer is thereby increased due, in part, to greater van der Waals interaction between adjacent lipid molecules. However, as noted previously[2], other factors which are dependent on the chemical configuration and physical state of the side chains may also play an important role. This was further revealed by preliminary experiments which indicated that dispersions of (dimyristoyl)-, (dipalmitoyl)- or (distearoyl)lecithin, in the presence of varying amounts of dicetyl phosphate and cholesterol, trapped little or no glucose when prepared at room temperature. These lecithins were examined because cholesterol has no significant effect on the cross-sectional area of (dipalmitoyl)- or (distearoyl)lecithin. DE GIER, MANDERSLOOT AND VAN DEENEN[10], however, demonstrated that it is possible to make liposomes from these lecithins at 37° or above. This can be explained by the liquid state of the paraffinic chains at higher temperatures[15]. We have also observed that liposomes prepared from (1-stearoyl-2-myristoyl)-lecithin with dicetyl phosphate alone did not trap any appreciable amount of marker at room temperature; however, when 33 and 50 mole % cholesterol was present, a measurable amount of glucose was trapped and only 4 % of the marker was released after incubation for 1 h at 41°. These results appear significant because the molecular area of this lecithin was reduced by cholesterol[2].

The question naturally arises whether the phenomenon described in the present paper has any biological importance. In this regard, some observations of STARR AND PARKS[16] appear extremely relevant. Cells of the yeast, *Saccharomyces cerevisiae*, perish when maintained at 40° or higher. STARR AND PARKS found that simultaneous addition of ergosterol and an unsaturated fatty acid (oleic) permitted the organism to survive

and grow at 40°. HASKINS[17] and SIETSMA AND HASKINS[18] have also reported that certain sterols maintain the viability of Pythium sp. at higher temperatures. On the basis of the above results, it seems possible that incorporation of these lipids into the fungal cell membrane (sterols *per se*, oleic acid as a constituent of phospholipids) was able to protect the membrane from the deleterious effect of elevated temperature. As demonstrated in the preceding paper[5], incorporation of cholesterol into liposomes increases their sensitivity to the polyene antibiotic, filipin. It is, therefore, also possible that these antifungal antibiotics may act by interfering with the ability of sterols to stabilize the bilayer configuration of lipids in some regions of the cell membrane.

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