Fluorescence Depolarization Studies of Phase Transitions and Fluidity in Phospholipid Bilayers. 2. Two-Component Phosphatidylcholine Liposomes†

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ABSTRACT: The fluorescence depolarization associated with the hydrophobic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene is used to monitor changes in fluidity accompanying the gel–liquid crystalline phase transition in phosphatidylcholine dispersions. In this way, the parameters of the phase transition are determined for both large, multilamellar liposomes and small, single-lamellar vesicles composed of three mixtures of phosphatidylcholines: dimyristoyl–dipalmitoyl, dimyristoyl–distearoyl, and dioleyl–dipalmitoyl. Phase diagrams for these mixed-lipid vesicles are constructed by plotting the delimiting temperatures of the phase transition vs. the lipid composition of the vesicle. The phase diagrams are interpreted to suggest that the miscibilities of the lipids studied are markedly different in small, single-lamellar vesicles and large, multilamellar liposomes. These results are discussed in terms of the effects of high curvature on the structure of biological membranes.

In the preceding paper (Lentz et al., 1976) we report studies of the fluidity and gel–liquid crystalline phase transition characteristics in small, single-lamellar vesicles and multilamellar liposomes prepared from pure synthetic phosphatidylcholines. In this paper we discuss these parameters for vesicles and liposomes prepared from binary mixtures of synthetic phosphatidylcholines. As in the preceding paper, depolarization of fluorescence of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] is used to monitor the apparent microviscosity in the hydrophobic core of the phospholipid bilayer and to characterize the gel–liquid crystalline phase transitions in these systems. Attention is focused on the utilization of the depolarization data to generate phase diagrams and to examine the phospholipid mixing characteristics in these binary systems.

Several recent papers have reported studies of lateral phase separation in multicomponent bilayers and have discussed the possible functional significance of this phenomenon in biological membranes (Phillips et al., 1970; Linden et al., 1973; Chapman et al., 1974; Shimshick and McConnell, 1973; Wu and McConnell, 1975). These studies have utilized multilamellar liposomes or undefined mixtures of these structures and small single-lamellar vesicles. Since it is now apparent that the phase transition characteristics in single-component bilayers are markedly different in small vesicles and multilamellar liposomes (Lentz et al., 1976; Suurkuusk et al., 1976), it is important to examine the phase characteristics in well-defined dispersions of both types. The possible physiological significance of the very small radius of curvature of the bilayer in small, single-lamellar vesicles has been discussed previously (Thompson et al., 1974; Lentz et al., 1976).

Materials and Methods

Preparation of Synthetic Phosphatidylcholines. The following L-phosphatidylcholines were prepared as previously described (Lentz et al., 1976): 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC); 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC); 1,2-distearoyl-3-*sn*-phosphatidylcholine (DSPC); 1,2-dioleyl-3-*sn*-phosphatidylcholine (DOPC); and 1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine (POPC). All phosphatidylcholines were judged to be better than 99% pure, based on thin-layer chromatography on silica gel G plates with a heavy loading of 2 μmol per spot. All saturated phosphatidylcholines were determined to contain greater than 99% of the expected fatty acid; DOPC contained 98% oleic acid in both positions 1 and 2. In the case of POPC, 99% of the fatty acid in position 1 was palmitic acid and 98% in position 2 was oleic acid.

Preparation of Phosphatidylcholine Dispersions. Both multilamellar liposomes and small, single-lamellar vesicles were prepared as described in the preceding paper (Lentz et al., 1976). To prepare mixed lipid dispersions, the component lipids in the desired proportions were first dissolved in $CHCl_3$ and this solvent was then completely removed under reduced pressure (colyophilized). The solid lipid mixture was used to prepare vesicles or liposomes. Dispersions were prepared between 5 and 10 °C above the gel–liquid crystalline transition of the highest melting component lipid and were maintained at this temperature until used. Addition of DPH to liposomes or vesicles were as described in the preceding paper (Lentz et al., 1976). The mole ratio of phosphatidylcholine to probe was maintained at 1000 to 1. Phospholipid concentrations were determined as inorganic phosphate by the Bartlett method (Bartlett, 1959).

Determination of Vesicle Composition. In order to construct accurately phase diagrams for small, single-lamellar vesicles, it was necessary to establish the actual composition of each dispersion after sonication and centrifugation since vesicles need not necessarily have the same composition as the colyophilized mixtures from which they were prepared. Vesicle composition was determined by fatty acid analysis. The phospholipids were first extracted by the Bligh and Dyer (1959) procedure. The fatty acids were then transmethylated using 3% HCl in dry methanol at 75 °C for 90 min, and the

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[1] Abbreviations used are: DPH, 1,6 diphenyl-1,3,5 hexatriene; POPC, 1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine (POPC); DMPC, DPPC, DSPC, and DOPC are 1,2-dimyristoyl-, 1,2-dipalmitoyl-, 1,2-distearoyl-, and 1,2-dioleyl-3-*sn*-phospatidylcholine, respectively.

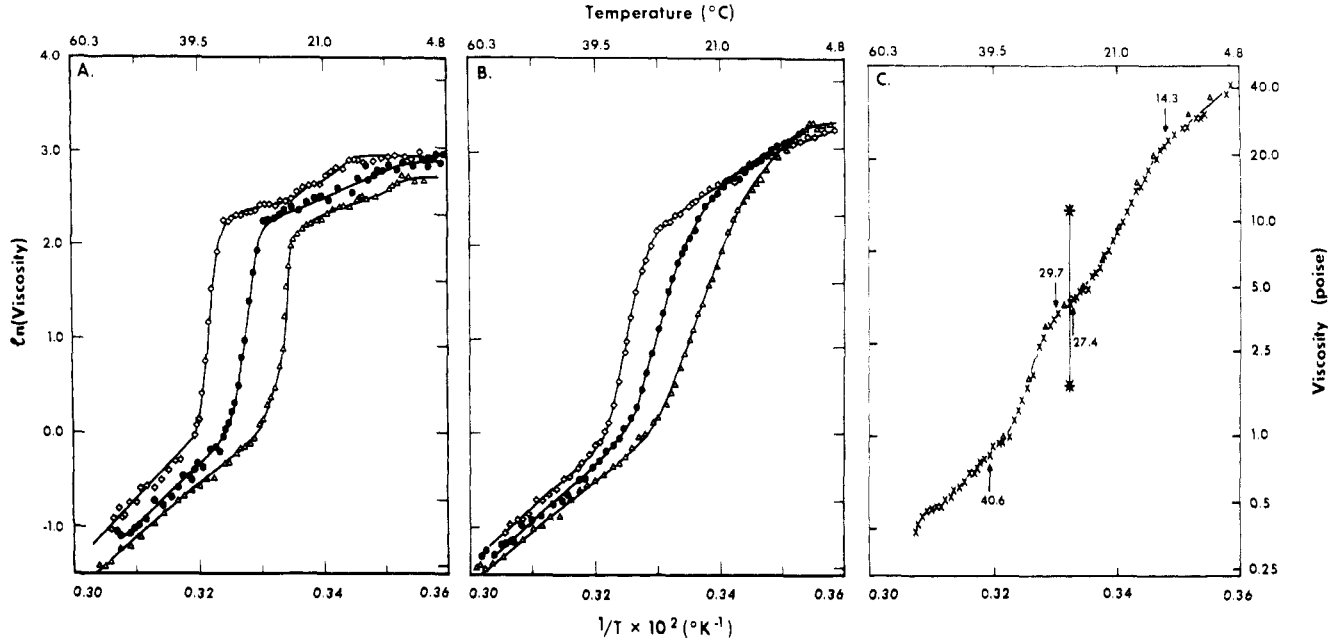

FIGURE 1: Plot of ln microviscosity vs. reciprocal of the absolute temperature for dispersions of DMPC/DPPC. (A) Multilamellar liposomes; (B) small, single-lamellar vesicles [(△) 83:17, (●) 50:50; (◇) 17:83 mol ratio]. (C) Mixture of small, single-lamellar vesicles composed of DMPC and similar vesicles composed of DPPC. the mixture of vesicles is comprised of equal amounts of each type. The arrows indicate the positions of the phase transitions characteristic of each vesicle type. The line marked by asterisks is at 28 °C.

resulting methyl esters were extracted into hexane and analyzed on a Shimadzu gas chromatograph equipped with a column of 10% Silar 10C on Gas Chrom Q (Applied Science). In all cases the measured fatty acid composition agreed within 2 mol % with the composition of the colyophilized mixture.

Fluorescence Measurements. DPH fluorescence depolarization measurements as a function of temperature and subsequent calculations of anisotropy and apparent microviscosity were carried out as described in the preceding paper (Lentz et al., 1976).

Results

Phase Transition Parameters. Delineation of the phase transition ranges for both small, single-lamellar vesicles and multilamellar liposomes was carried out as described in the preceding paper (Lentz et al., 1976). In all cases the data were fit by lines having slopes with correlation coefficients better than 0.992 and standard deviations less than 2%.

Arrhenius plots of the apparent microviscosity of mixtures of DMPC and DPPC are presented in Figure 1. The data for large multilamellar liposomes are shown in Figure 1A and for small, single-lamellar vesicles in Figure 1B. By way of contrast, Figure 1C is the Arrhenius plot for the apparent microviscosity of an equimolar mixture of small, single-lamellar vesicles formed from DMPC and similar vesicles formed from DPPC. Two phase transitions are evident, one corresponding to pure DPPC, and the other to pure DMPC. Labeled arrows delimit the temperature extremes of these transitions, as summarized in Table II of the preceding paper (Lentz et al., 1976). The line delineated by asterisks indicates the temperature of the DPH exchange experiment discussed below. The characteristics of this mixture of vesicles are in marked contrast to the characteristics of vesicles prepared from a colyophilized mixture of these two phospholipids as shown in Figure 1B. Similar differences were observed between multilamellar liposome samples prepared from colyophilized lipid mixtures and from mixtures of two different liposome populations. These results clearly show that in colyophilized binary systems the lipids mix within individual bilayers rather than segregating into the bilayers of different liposomes.

Arrhenius plots similar to Figures 1A and 1B are shown in Figure 2 for large multilamellar liposomes prepared from (A) DMPC/DSPC and (C) DOPC/DPPC and for small single-lamellar vesicles prepared from (B) DMPC/DSPC and (D) DOPC/DPPC. In both Figures 1 and 2, data for only three representative mixtures are shown, although data were obtained for at least seven different compositions in order to generate each phase diagram presented below.

The following characteristics of the data presented in Figures 1A, 1B, and 2 are noteworthy. First, the Arrhenius plots for multilamellar liposomes composed of DMPC/DPPC mixtures show both low- and high-temperature transitions which are very reminiscent of the behavior of liposomes prepared from the pure components (Figure 1A). Multilamellar liposomes formed from mixtures of DMPC/DSPC and DOPC/DPPC, however, show two transitions only for a few compositions. Most compositions show only a single well-defined transition (Figures 2A and 2C). The apparent absence of a low-temperature transition may be because this transition is obscured by a broadened high-temperature transition in these systems. Second, the effects of compositional variations on the phase transition characteristics of both small, single-lamellar vesicles and multilamellar liposomes are least apparent for DMPC/DPPC mixtures, but become increasingly more apparent for DMPC/DSPC and DOPC/DPPC mixtures, respectively. Third, comparison of Figures 1A, 2A, and 2C with Figures 1B, 2B, and 2D clearly shows that the effects of compositional variations are more apparent in small, single-lamellar vesicles than in multilamellar liposomes comprised of the same two phospholipids.

Partitioning of DPH between Phospholipid Phases. The use of DPH to monitor the gel–liquid crystalline phase transition is based on the assumption that the fluorescence probe is equally distributed between gel and liquid crystalline phases in the temperature range of the transition where both phases coexist. If the bilayer system consists of two phases, and the

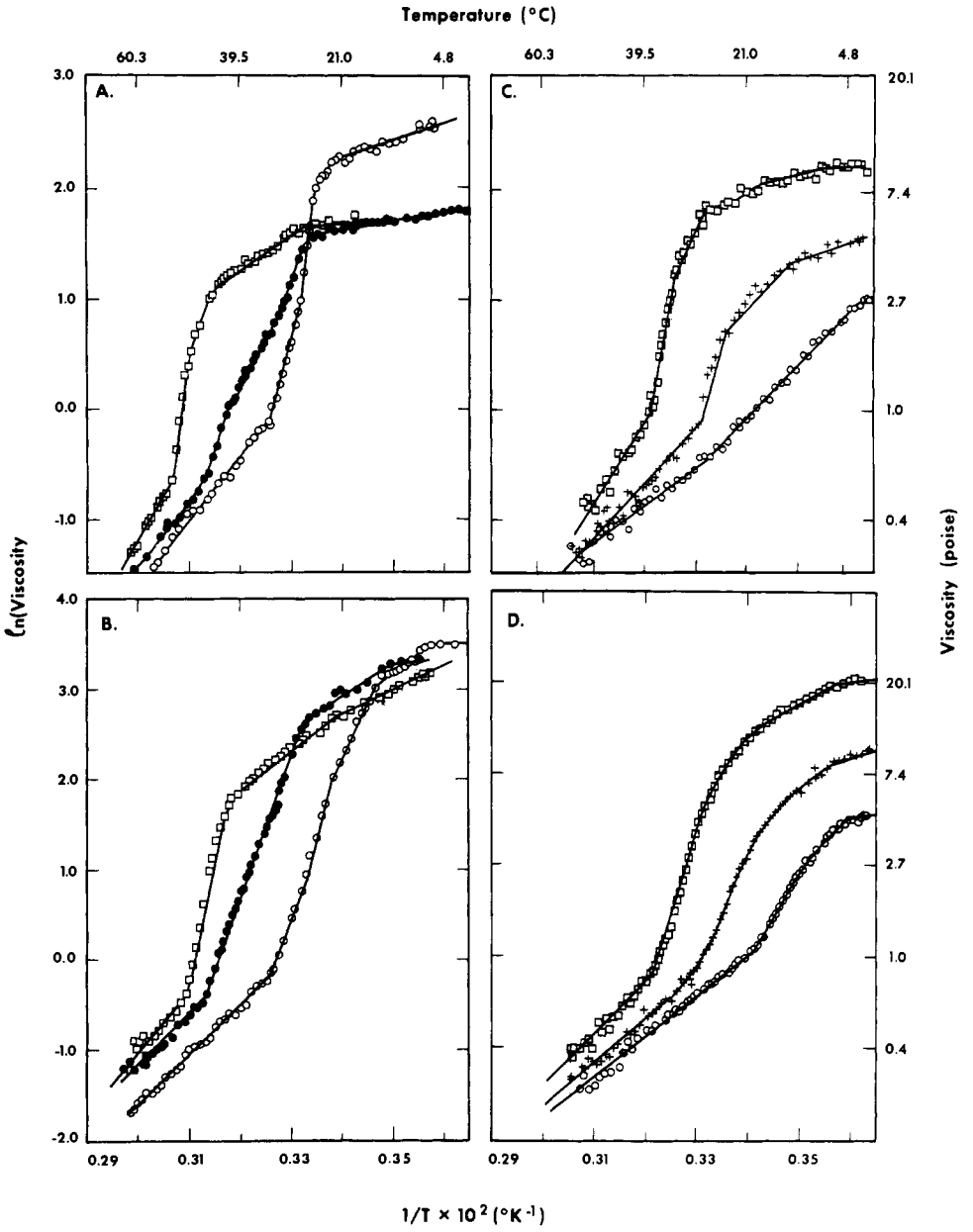

FIGURE 2: Plot of ln microviscosity vs. reciprocal of the absolute temperature for dispersions of DMPC/DSPC. (A) Multilamellar liposomes; (B) small, single-lamellar vesicles, and dispersions of DOPC/DPPC; (C) multilamellar liposomes; (D) small, single-lamellar vesicles [(○) 83:17; (●) (+) 50:50; (□) 17:83 mol ratio].

probe is equally distributed between them, the measured anisotropy is a simple average of the anisotropy of the probe in each phase. If, however, the probe shows a strong preference for one phase, the observed anisotropy will be strongly weighed in favor of the signal from this phase. To the extent this occurs, the resulting profile of the phase transition is shifted toward either low temperature (gel phase preferred) or high temperature (liquid crystalline phase preferred).

We have examined the validity of this assumption by studying the partitioning of DPH between a mixture of DPPC and DMPC vesicles at 28 °C which is below the liquid crystalline phase transition temperature of DPPC, but above that of DMPC. The fluorescence of DPH in each vesicle species was measured at this temperature. One vesicle species containing DPH was then mixed with a known amount of the other vesicle species containing no DPH, and the change in fluorescence depolarization was followed as a function of time. A constant value of the anisotropy was achieved in less than 30 s, the minimum time required for performing the measurement. This result indicates that the transfer of DPH between vesicles occurs within a few seconds. Two such experiments were performed. In the first case, the DPH was initially incorporated into DMPC vesicles and then allowed to transfer to DPPC vesicles. In the second case, the DPH was initially in the DPPC vesicles and transferred to DMPC vesicles (see Table I). Proof that the observed change in fluorescence depolarization was due to dye transfer rather than to vesicle fusion and subsequent lipid mixing is given in Figure 1C. This figure is the temperature scan for the apparent microviscosity as observed in the vesicle mixtures resulting from the two probe distribution experiments. The curve shows two phase transitions, one characteristic of pure DPPC, the other of pure DMPC. Comparison of the data in Figures 1A, 1B and 1C clearly indicates that vesicle fusion did not occur. The results of these two DPH

TABLE I: Partitioning of DPH between Gel and Liquid Crystalline Phases.

Sample	r	τ	C^a	$X_{DMPC}{}^b$	$X_{DPPC}{}^b$	Z_{DMPC}	Z_{DPPC}	f_{DMPC}	f_{DPPC}	$K_{m/p}$
DMPC + DPH	0.127 ±0.003	9.3 ±0.1	0.613 ±0.008	1.0	0.0					
DPPC + DPH	0.288 ±0.006	10.3 ±0.1	0.429 ±0.008	0.0	1.0					
(DMPC + DPH), 2 ml + DPPC, 2 ml	0.196 ±0.004	9.8 ±0.1	0.521[c]	0.59 ±0.03	0.41 ±0.02	0.57 ±0.02	0.43 ±0.02	0.60 ±0.02	0.40 ±0.02	1.03 ±0.04
(DPPC + DPH), 2 ml + DMPC, 2 ml	0.199 ±0.004	9.6 ±0.1	0.521[c]	0.59 ±0.03	0.41 ±0.02	0.55 ±0.02	0.45 ±0.02	0.58 ±0.02	0.42 ±0.02	0.96 ±0.04

[a] Concentration of total phospholipids (μmol of lipid per ml). [b] Mole fraction of DMPC or DPPC in the total lipid suspension. [c] Average of DMPC and DPPC concentrations since equal volumes of DMPC and DPPC suspensions were mixed. [d] Error limits for the experimental quantities are estimated standard deviations; not enough data exist to define exact statistical standard deviations. Error limits for the derived quantities (Z, f, k) are calculated from those assigned to the experimental quantities.

partitioning experiments are presented in Table I. The partition coefficient of DPH between these two lipid bilayer phases was calculated as outlined below.

For a fluorescent probe partitioned between two environments of different fluidity which exist for a time which is long relative to the fluorescent decay time of the probe, the observed fluorescence will be a composite of the fluorescence from the probe in the two environments:

$$I_{\parallel} = f_1 I_{\parallel}(1) + f_2 I_{\parallel}(2) = \langle I_{\parallel} \rangle_f$$
$$I_{\perp} = f_1 I_{\perp}(1) + f_2 I_{\perp}(2) = \langle I_{\perp} \rangle_f \quad (1)$$

Here f_1 and f_2 are the mole fractions of probe in environments 1 and 2, respectively. $I_{\parallel}(1)$ and $I_{\perp}(1)$ are the parallel and perpendicular intensities that would result if all the probe were in environment 1, and $I_{\parallel}(2)$ and $I_{\perp}(2)$ are the corresponding values for environment 2 (assuming, of course, that excimer formation, energy transfer, and other concentration-dependent effects are not operative). $\langle\ \rangle_f$ denotes an f-weighted average. Since

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (2)$$

we obtain for the composite system

$$r = Z_1 r_1 + Z_2 r_2 = \langle r \rangle_Z \quad (3)$$

with

$$Z_1 = [f_1/(f_1 + f_2 F_2/F_1)] \quad (4)$$

and,

$$r_1 = [I_{\parallel}(1) - I_{\perp}(1)]/[I_{\parallel}(1) + 2I_{\perp}(1)] \quad (5)$$

Here $\langle r \rangle_Z$ denotes a Z-weighted average. The quantity Z may be thought of as the mole fraction of probe in environment 1, adjusted for the relative intensity of the fluorescence in environments 1 and 2 (F_1 and F_2, respectively). When only dynamic quenching occurs, F_2/F_1 equals the ratio of lifetimes in the different environments (Weber, 1953). In general, then, it can be shown that the intensity adjusted mole fraction is given by:

$$Z_j = f_j[\sum_i f_i(\tau_i/\tau_j)] \quad (6)$$

Here, τ_i/τ_j is the ratio of lifetimes. The sum is over all environments (other than the jth) into which the probe may partition. It also may be shown that

$$\sum_j Z_j = 1 \quad (7)$$

The Z values for DPH in Table I have been calculated using eq 3 and 7 and the experimentally observed r values listed in Table I. The mole fraction of DPH in each of the bilayer environments (f_{DMPC} and f_{DPPC}) has thus been computed using eq 6 and 7 and the experimental lifetimes (τ of Table I). In order to obtain the partition coefficient of DPH between DMPC and DPPC vesicles, the f values must be corrected for the mole fraction (X_i) of each of the lipids present in the mixture:

$$K_{m/p} = (f_{DMPC}/X_{DMPC})/(f_{DPPC}/X_{DPPC}) \quad (8)$$

The values of $K_{m/p}$ calculated from the two parallel experiments are given in the last column of Table I and clearly demonstrate that DPH does not partition preferentially into either the liquid crystalline or the gel phase. Thus, under conditions where phases coexist in a single bilayer, the observed steady-state anisotropy of DPH fluorescence is the Z-weighted average of the anisotropies of each phase. Indeed, if the lifetimes of DPH are not greatly different in the two phases, the anisotropy, and thus the microviscosity, will directly reflect the relative amounts of the two phases. This is the situation that exists for the systems listed in Table I. It is apparent that values of f are approximately equal to the corresponding values of Z.

Discussion

Phase Diagrams and Lipid Mixing. Using the procedures outlined in the Results section, the delimiting temperatures of the phase transition were determined for both large multilamellar liposomes and small, single-lamellar liposomes prepared from several compositions of the three lipid mixtures already discussed.[2] These delimiting temperatures were plotted vs. composition to generate the phase diagrams shown in Figures 3A, 4A, and 5A (large, multilamellar liposomes) and Figures 3B, 4B, and 5B (small, single-lamellar vesicles). In-

[2] The phase behavior of two-component liposomes may involve sharp, well-defined phase changes nearly identical with those of single-component liposomes, or gradual, poorly defined phase changes involving only a portion of the lipids in the bilayer. To describe phase changes in a multicomponent lipid bilayer, McConnell and co-workers (McConnell et al., 1972; Shimshick and McConnell, 1973) have coined the term "phase separation". This applies to the separation from a homogeneous liquid phase of a solid phase rich in one component of the mixture (McConnell et al., 1972). So defined, this term would seem inappropriate to describe the phase changes in a bilayer in which the lipid mixing is imperfect even in the liquid-crystalline state. For this reason, only the term "phase transition" is used in this paper. If the term "phase separation" is more appropriate, this should be clear from the description of the lipid mixing properties of each system studied.

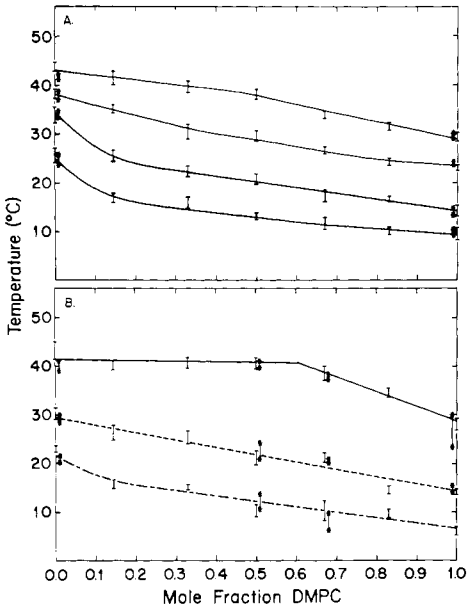

FIGURE 3: Phase diagrams for dispersions of DMPC/DPPC mixtures. (A) Large, multilamellar liposomes; the two upper lines delimit the high-temperature phase transition range, the lower lines the low-temperature transition range. (B) Small, single-lamellar vesicles. The asterisked segments represent repeated data points.

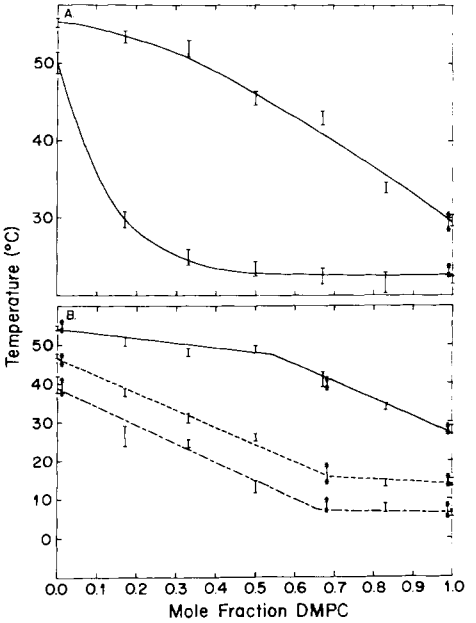

FIGURE 4: Phase diagrams for dispersions of DMPC/DSPC mixtures. (A) Large, multilamellar liposomes; (B) small single-lamellar vesicles. * as in Figure 3.

terpretation of phase diagrams generated in this way for bilayer systems must be made with caution. Lee (1975a) has discussed this problem in some detail.

The phase diagram for DMPC and DPPC in multilamellar liposomes is shown in Figure 3A. The compositional behavior of both the high- and low-temperature (with cross-hatching) transitions were obtained, but the range of the low-temperature transition was less well-defined than the range of the high-temperature transition. The shape of the phase diagram for the high-temperature transition suggests that these two very similar lipids mix nearly ideally both in the gel and liquid crystalline phases in the bilayers of multilamellar liposomes. The phase diagram for this system has been previously obtained by Shimshick and McConnell (1973) using partitioning of Tempo spin label. These authors represented the phase transition for the pure lipids as being infinitely sharp, while that for the 50/50 mol ratio mixture was shown to have a spread of about 5 °C. However, the primary data of Shimshick and McConnell appear to agree with our data presented in the preceding paper (Lentz et al., 1976) and with available calorimetric data (Hinz and Sturtevant, 1972; Suurkuusk et al., 1976) in showing the high-temperature phase transition of pure phosphatidylcholine vesicles to be of substantial breadth. A similar point has recently been made by Lee (1975b) in a fluorescence probe study using chlorophyll a as the fluorophore. A calorimetric study of this system, although subject to uncertainties in interpretation due to instrumental difficulties, yielded a phase diagram in qualitative agreement with ours (Chapman et al., 1974).

The composition dependence of the low-temperature transition for DMPC/DPPC mixtures is also recorded in Figure 3A. This binary mixture was the only one for which this transition could be consistently observed at all compositions. As was the case for the high-temperature transition, this transition showed little, if any, broadening in the mixed-lipid vesicles. The low-temperature transition was separated from the main transition by about the same temperature span at all compositions above 10 mol % DMPC. The general characteristics of the phase diagram in DMPC/DPPC mixtures suggest that the mixing of these two phospholipids in multilamellar bilayers is essentially ideal.

Figure 4A is the phase diagram for the high-temperature phase transition in multilamellar liposomes composed of mixtures of DMPC and DSPC, two phosphatidylcholines with saturated acyl chains differing in length by four carbon atoms. This phase diagram is markedly different from that shown in Figure 3A obtained for two phosphatidylcholines with saturated chains differing in length by only two carbons. Figure 4A indicates that DMPC and DSPC mix nonideally. The horizontal section of the solidus curve (lower line) in the mole fraction range 0.3 to 1.0 suggests that in this range two solid phases of different composition coexist. This same conclusion was reached by Shimshick and McConnell (1973) based on Tempo partitioning, although the detailed shape of their phase diagram was somewhat different from that in Figure 4A. Also, since Shimshick and McConnell presented no data in the range 0 to 0.38 mol fraction DMPC, it is not possible to specify the concentration at which the horizontal solidus line terminates. However, the fact that it must be within this range is consistent with Figure 4A. An earlier scanning calorimetric study (Phillips et al., 1970), however, indicated that the horizontal segment terminates below 0.22 mol fraction DMPC. This is clearly inconsistent with our results.

It is interesting to note that in this binary system at least two, and in one case three, distinct regions of near linearity are observed through the region of the phase transition in both the microviscosity Arrhenius plots and the anisotropy vs. temperature plots (see Figure 2A). The slopes and correlation coefficients of these lines are summarized in Table II. Similar behavior within the range of the transition was observed only for lipid mixtures in which the shape of the phase diagram suggested gel phase immiscibility (see results for DMPC/DSPC and DOPC/DPPC small, single-lamellar vesicles discussed below). It is also interesting to note the symmetry displayed by the data in Table II. With vesicles rich in DMPC the most rapid increase in microviscosity occurs in the low-temperature portion of the phase transition temperature range,

TABLE II: Slopes of Linear Sections within the Phase Transition of Large DMPC/DSPC Vesicles.

Mole	Line 1[a]			Line 2[b]			Line 3		
Fraction DMPC	Slope 1000	Corr Coef[d]	Range (°C)	Slope 1000	Corr Coef[d]	Range (°C)	Slope 1000	Corr Coef[d]	Range (°C)
1.0				97.5 ± 8.9	0.965	24.2-24.8			
0.83	17.0 ± 0.5	0.998	28.5-32.5				35.2 ± 1.4	0.997	25.9-28.5
0.67	10.7 ± 0.3	0.995	31.6-40.8				21.7 ± 0.6	0.995	26.2-31.6
0.50	15.1 ± 1.1	0.992	42.1-45.5	8.3 ± 0.2	0.997	30.7-39.2	14.6 ± 0.9	0.993	28.1-31.2
0.33	19.7 ± 0.7	0.996	46.1-50.2				6.7 ± 0.1	0.997	28.9-43.3
0.17	37.4 ± 1.4	0.998	50.6-53.2				3.1 ± 0.1	0.988	29.5-42.7
0.00				172.6 ± 20.1	0.987	53.4-54.6			

[a] High-temperature line. [b] Intermediate-temperature line; or single line in case of pure lipids. Low-temperature line. [d] Correlation coefficient.

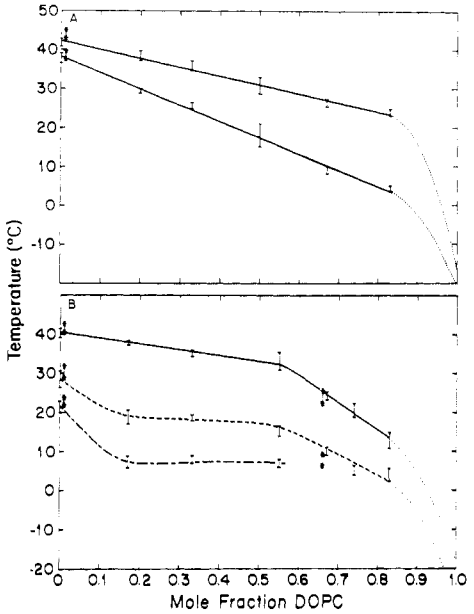

FIGURE 5: Phase diagrams for dispersions of DOPC/DPPC mixtures. (A) Large, multilamellar liposomes; (B) small, single-lamellar vesicles. * as in Figure 3.

while the opposite is true for vesicles rich in DSPC. Indeed, in the 50/50 mixture, the low- and high-temperature ranges have equal slopes and are separated by a region of intermediate slope. This suggests that, at least within the range of the phase transition, there are regions rich in one or the other of these two lipids. Regions rich in DMPC would then determine the nature of the low-temperature portion of the phase transition, while regions rich in DSPC should determine the nature of the high-temperature portion of the transition. Thus, for vesicles rich in DMPC, the phase transition would be most pronounced in the low-temperature region (see line 3 of Table II), while the opposite would be true of vesicles rich in DSPC (see line 1 of Table II). These results support further the interpretation of the DMPC/DSPC multilamellar liposomes phase diagram in terms of incomplete mixing of the lipids.

The phase diagram for mixtures of DOPC and DPPC in large multilamellar liposomes is presented in Figure 5A. This figure clearly indicates that a cis double bond in the acyl chains of one component markedly alters the mixing properties of these lipids. Oleic acid (18:1.9) and palmitic acid (16:0) differ by only two carbon atoms in length, as do myristic (14:0) and palmitic acids, the mixture of Figure 3A. However, DOPC and DPPC obviously do not mix in the apparently ideal manner of DMPC and DPPC (Figure 3A). In contrast to the very slight effect of composition on the breadth of the transition in the latter system, Figure 5A shows increased broadening of the transition with increasing content of DOPC in multilamellar liposomes, up to 0.83 mol fraction DOPC. Above this concentration, the low-temperature limit of the phase transition is very close to or below 0 °C, and the transition becomes so poorly defined that it is difficult to determine the limits. The dotted line in Figure 5A is drawn on the basis of the results of Phillips et al. (1970), who demonstrated that hydrated DOPC (50% water, w/w) has a gel-liquid crystalline phase transition at −22 °C. A recently published differential calorimetry scan of DOPC multilamellar vesicles (De Kruijff et al., 1975) suggested that a phase transition occurs in the neighborhood of −20 °C. Within these limits, the shape of the phase diagram of Figure 5A (especially the rapid drop in transition temperature as pure DOPC is approached) clearly implies that DOPC and DPPC do not mix ideally. Such a result was not observed for any mixture of two saturated lecithins of similar chain length incorporated into large, multilamellar liposomes.

A calorimetric study of multilamellar liposomes composed of 1:1 mixture of DOPC and DPPC (i.e., one point on the phase diagram of Figure 5A) showed two phase transitions, one in the region of the DPPC transition and one in the region of the DOPC transition (Phillips et al., 1972). A transition in the range of −20 °C must be measured either in a buffer containing a high concentration of some organic antifreeze or in ice. Within the uncertainty imposed by these conditions, however, it appears that these phosphatidylcholines segregate into regions rich in DPPC and DOPC, respectively. Thus, the phase diagram of Figure 5 corresponds only to the transition of the DPPC-rich regions, that of the DOPC-rich regions being inaccessible without inclusion of antifreeze in the aqueous medium. These observations suggest that the unusual shape of the apparent solidus line of Figure 5A should be interpreted in terms of coexisting gel and liquid crystalline phases at temperatures below this line. As noted above, the fact that the liquidus line of Figure 5A does not have a more negative slope at high DPPC mol fraction does suggest that domains rich in each of the lipids may exist in the liquid crystalline state. The observation of two phase transitions in 1:1 liposomes (Phillips et al., 1972) would seem to support this conclusion.

Phase diagrams derived for small, single-walled vesicle systems are quite different from those for large multilamellar liposome dispersions shown in Figures 3A, 4A, and 5A. As is evident in Figures 3B, 4B, and 5B, the phase diagrams are more complex. The phase transitions are broader in single-lamellar

vesicles, and at least two straight lines are needed to fit the data in microviscosity Arrhenius plots at low temperatures. For this reason, the phase diagrams in Figures 3B, 4B, and 5B have two solidus lines. The higher temperature line delimits the main phase transition in small vesicles. The significance of the lower temperature line is unclear.

Figure 3B is the phase diagram derived from the fluorescence data for small single-lamellar vesicles composed of DMPC/DPPC mixtures. Its most striking feature is the nearly horizontal liquidus line, suggesting that these two very similar lipids mix nonideally in the liquid-crystalline state. This is particularly surprising since these two lipids mix nearly ideally in the multilamellar liposomes described above. There are several possible explanations for this behavior: (1) the vesicles formed by the sonication process are a compositionally heterogeneous population, with some vesicles very rich in DPPC and others very rich in DMPC; (2) the lipids are distributed unequally between the nonequivalent inner and outer monolayers of the small vesicles; (3) because of the special packing requirements introduced by the small radius of curvature of sonicated vesicles (Sheetz and Chan, 1972; Thompson et al., 1974; Lentz et al., 1976), these two similar lipids really do mix imperfectly nearly the point of immiscibility, in the inner or outer monolayer of the small vesicle bilayer. The first explanation seems unlikely, for three reasons. First, complete or nearly complete segregation of the two lipids into separate vesicle populations would have resulted in an Arrhenius plot of much different shape (i.e., as in Figure 1C). Second, determination of the fatty acid composition of the sonicated vesicle preparations indicated no preference for either lipid of a mixture to appear in large as opposed to small liposomes. If there were no preferential partitioning of lipids between vesicles of different radii of curvature, it would be difficult to imagine a reason for preferential partitioning between vesicles of very similar radii of curvature. Third, the existence of a population of vesicles composed almost entirely of DPPC would necessarily imply a coexisting population composed almost exclusively of DMPC. This should result in an apparent nonmixing in the gel phase as well as in the liquid-crystalline phase. However, no such behavior is observed. For a similar reason, the second explanation in unlikely, that is, enrichment of the other monolayer with DMPC necessarily would imply a concomitant enrichment of the inner monolayer with DPPC. No evidence for this is observed. Thus, it appears most likely that the shape of Figure 3B indicates that packing constraints in these small vesicles cause DMPC and DPPC to mix nonideally in the liquid crystalline state. That such imperfect mixing occurs in the liquid crystalline phase and not the gel phase is surprising. On the other hand, it is, in fact, reasonable to expect that the extended, all-trans configuration of the phospholipid acyl chains in the gel state (Seelig and Seelig, 1974) would be less affected by the unusual packing requirements of a small radius of curvature vesicle than would the disordered, gauche-containing acyl chains of the liquid crystalline state.

The phase diagram for mixtures of DMPC and DSPC incorporated into small single-lamellar vesicles is shown in Figure 4B. Again, there is a clear indication of nonideal mixing in the liquid crystalline state in these vesicles which is not observed in multilamellar liposomes. As is the case with multilamellar liposomes, the phase diagram of this lipid mixture also suggests immiscibility in the gel phase. However, immiscibility occurs in small vesicles only at concentrations of DMPC above 0.6 mol fraction, whereas in multilamellar liposomes this occurs above 0.3 mol fraction (Figure 4A). It is interesting to note that for single-lamellar (as for multilamellar liposomes) the Arrhenius plots of the apparent microviscosity for 0.83 and 0.67 mol fraction DMPC show two distinct straight lines in the phase transition region. This suggests two phases of different composition in the gel state. In summary, small, single-lamellar vesicles composed of DMPC/DSPC show the same gel-phase immiscibility as do multilamellar liposomes, but over a narrower range of composition. In addition, they display a nonideality of mixing in the liquid crystalline phase not observed in the multilamellar liposomes. The interpretation of this behavior is similar to that suggested for the results obtained for small vesicles composed of DMPC/DPPC; the disordered acyl chains of the liquid crystalline phase mix considerably less ideally, while the ordered acyl chains of the gel phase mix more nearly ideally under the packing constraints imposed by the small radius of curvature of a single-lamellar vesicle.

The DOPC/DPPC phase diagram shown in Figure 5B illustrates the effect of a cis double bond in the acyl chains of one phospholipid component in the small, single-lamellar vesicle system. The 9,10 double bond causes a fixed gauche-like kink at this point in the acyl chain. By analogy to the behavior of large multilamellar liposomes, this phase diagram probably represents the phase behavior of only the DPPC-rich regions of the lipid bilayer. Although there is no direct evidence in small, single-bilayer vesicles, another phase change probably occurs in the range of −20 °C, which is presumed to be the phase-transition range for pure DOPC small vesicles. The phase diagram in Figure 5B is interpreted in light of these assumptions. As in the case of the saturated systems already discussed, the shape of the liquidus line in Figure 5B suggests nonideal mixing of DPPC and DOPC in the liquid crystalline phase. The existence of a nearly horizontal section of this line below 0.55 mol fraction DOPC suggests liquid crystalline phase immiscibility even more convincingly than in compositionally similar multilamellar liposomes (Figure 5A). It is interesting that the solidus line in Figure 5B also has a distinctly horizontal section (0.17 to 0.55 mol fraction DOPC). This probably reflects a limited miscibility of DPPC and DOPC in the DPPC-rich gel state. This phenomenon was not observed for DPPC/DOPC mixtures in large, multilamellar liposomes (Figure 5A). The Arrhenius plots of microviscosity for mole fractions of DOPC from 0.17 to 0.55 are fit well by two straight lines in the temperature range of the phase transitions (correlation coefficient of the slope > 0.986; standard deviation from a straight line < 6.5%). This is analogous to the behavior of the other systems studied for which gel phase immiscibilities were noted (DMPC/DSPC multilamellar liposomes and small vesicles). Thus, the phase diagram in Figure 5B is consistent with the hypothesis that, in small radius of curvature bilayers, lipids with fully extended acyl chains (those of saturated lipids below the phase transition) mix more nearly ideally than do lipids with kinked acyl chains (unsaturated chains, or any chain above the phase transition).

It is worthwhile noting that the small vesicle, mixed lipid systems so far discussed are examples of lipid bilayers for which the term "phase separation" is not an entirely appropriate description of the gel–liquid crystalline phase transition. This is so because lipid immiscibility seems often to be associated with the liquid crystalline phase of the small radius of curvature bilayers, which should result in some degree of phase separation (or lipid-domain formation) even above the phase transition temperature. It is this liquid crystalline phase bilayer heterogeneity (rather than gel phase heterogeneity) which results in the phase transition broadening in mixed-lipid, small vesicles. It is not clear whether this heterogeneity should increase on cooling through the phase transition, as implied by

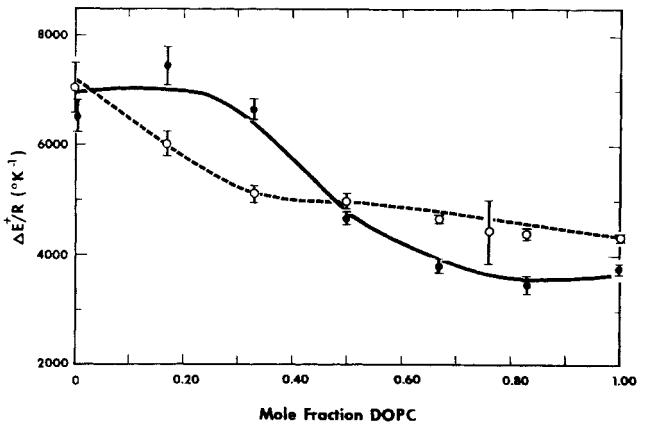

FIGURE 6: Plot of activation energy vs. mole fraction of DOPC for DOPC/DPPC mixtures. (○) Small, single-lamellar vesicles; (●) large, multilamellar liposomes.

the common use of the term "phase separation" (McConnell et al., 1972).

The Activation Energy of the Apparent Microviscosity. Changes in the temperature dependence of the microviscosity have been observed as a function of fatty acid composition in the vesicle and liposome systems studied. For the purpose of discussion, it is convenient to note that the activation energy of the microviscosity (ΔE^{+}) may be defined as R, the gas constant, times the slope of the Arrhenius plot of the apparent microviscosity. In the theory of Eyring (1936), this is the activation energy of the rate determining step of viscous flow. More recently, Shinitzky (personal communication) has related the activation energy to a molecular volume. Although the exact molecular interpretation of this quantity remains obscure, it is nevertheless a useful relative structural parameter. As noted in the preceding paper (Lentz et al., 1976), the activation energy of the microviscosity was less for DOPC than for any of the saturated phosphatidylcholines incorporated into either small, single-lamellar vesicles or multilamellar liposomes. In both of these systems comprised of mixtures of saturated phosphatidylcholine, ΔE^{+} has an approximately constant value of 12.5 ± 1.5 kcal/mol. However, in both small vesicles and multilamellar liposomes comprised of DOPC/DPPC, ΔE^{+} varies from about 8 kcal mol^{-1} deg^{-1} for pure DOPC to 14 kcal mol^{-1} for pure DPPC as shown in Figure 6.

Summary and Conclusions

The results presented in this paper indicate that the mixing of phosphatidylcholine molecules within a bilayer is affected not only by the nature of the acyl chains, but also by the geometry of the bilayer. Two different phosphatidylcholine species mix less ideally in small, single-walled vesicles which have a radius of curvature of about 120 Å than in multilamellar liposomes of very large radius of curvature. The phase diagrams obtained for small vesicles are most simply interpreted in terms of nonideal mixing of components in the plane of the bilayer as opposed to nonideal mixing across the plane of the bilayer. In most cases, too, the range of temperatures for which a gel phase exists in equilibrium with a liquid crystalline phase is increased in small vesicles relative to large liposomes. Thus, the effect of bending a lipid bilayer to give a small radius of curvature is to increase the range of temperature and composition over which different phases may coexist in the plane of the bilayer. There has been considerable speculation recently about the importance of lateral phase separation of lipids in biomembranes (Shimshick and McConnell, 1973; Linden et al., 1973). If these speculations are correct, then our results suggest that regions of high curvature in biomembranes may be associated with in-plane formation of lipid domains of differing composition. Of even greater significance is the fact that these results suggest the possibility of lateral segregation of lipids into different domains in highly curved bilayers even *above* the gel-liquid crystalline phase transition. This is not "lateral phase separation" in the sense originally used by McConnell et al. (1972) since it is induced by changes in bilayer curvature rather than by passage below the liquid crystalline phase transition temperature. This phenomenon would more appropriately be referred to as lateral lipid domain formation. Since most biomembranes exist at temperatures above the gel-liquid crystalline phase transition of the component lipids, this phenomenon provides a means of introducing lateral inhomogeneities into the bilayers of biomembranes. Such inhomogeneities should result in the same special compressibility properties that have been speculated to occur in bilayers undergoing lateral phase separation (Shimshick and McConnell, 1973; Linden et al., 1973).

The results of this study also suggest a role for unsaturated fatty acids in the lipids of biomembranes. First, our studies with systems composed of DOPC and DPPC (together with the study of Phillips et al., 1972) have demonstrated that introduction of double bonds into the fatty acid chains of phosphatidylcholines results in decreased miscibility of the bilayer lipids in both small vesicles and multilamellar liposomes. This increases the possibility of lateral compositional inhomogeneities in the bilayer. Second, introduction of unsaturated phosphatidylcholines into bilayers markedly lowers the activation energy of the microviscosity in the temperature range above the phase transition. In practical terms, this would have the effect of moderating changes in membrane fluidity that occur with changes in temperature.

Comparison of POPC bilayers with bilayers formed from a 1:1 mixture of DOPC and DPPC has further shown that the particular arrangement of unsaturated fatty acids in and among the phosphatidylcholine molecules of the bilayer is also important in predicting the effect of unsaturated fatty acids on membrane structure. Thus, POPC bilayers undergo phase transitions at much lower temperatures than the corresponding 1:1 DOPC/DPPC bilayers. However, at temperatures above the phase transition, POPC systems have microviscosities and activation energies very similar to those of DOPC/DPPC systems.

Thus, a cell should have great variability in adjusting the properties of its membranes, even if the membrane bilayer were to consist solely of phosphatidylcholine. Introduction of unsaturated fatty acids would produce more fluid membranes, but arrangement of these fatty acids both on one phosphatidylcholine molecule would allow for a greater possibility of lateral bilayer inhomogeneities occurring at physiological temperatures. Inclusion of phospholipids with much different fatty acid moieties would also increase the chance of lateral phase separation. Finally, even if the membrane as a whole did not undergo lipid lateral phase separation, small regions of high curvature could be formed in which lateral separation of component lipids into domains would be more probable.

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Effects of Iodination of Tyrosyl Residues on the Binding and Action of Glucagon at Its Receptor†

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ABSTRACT: The binding and action of glucagon at its receptor in hepatic plasma membranes have been compared, as a function of pH, with that of glucagon containing iodotyrosyl residues. Iodinated glucagon, at pH 7.0 and below, binds to the receptor and activates adenylate cyclase with an affinity about threefold higher than that of native glucagon. At pH 8.5, the affinity of the receptor for native glucagon is the same as that seen at pH 7.0. However, iodinated glucagon binds with a lowered affinity with increasing pH. The decreased affinity of the iodinated hormone correlates with ionization of the iodotyrosyl phenoxy group, which has a pK_a of 8.2. It is suggested that the decreased affinity is actually due to the inability of the ionized iodoglucagon to bind to the receptor. The relative potency of native and iodoglucagon will depend, therefore, on the concentrations of ionized and un-ionized species of iodoglucagon, which in turn depend on the pH of the medium. We conclude that incorporation of iodine atoms in the tyrosyl residues of glucagon has two major effects: (i) the iodine atom increases hydrophobic interaction of the hormone with the receptor and (ii) ionization of the phenoxy groups results in the loss of biological activity possibly as the result of loss of hydrogen bonding capability. Thus, the tyrosyl residues in glucagon are critically involved in the function of the hormone.

There are conflicting reports in the literature concerning the effect of iodination of glucagon on its binding and action on receptor-mediated adenylate cyclase systems. While enhanced biological potency has been reported for iodoglucagon (Bromer et al., 1973; Desbuquois, 1975), in two separate studies we were unable to detect any difference in the potency of native and monoiodoglucagon (Rodbell et al., 1971; Lin et al., 1975). The reasons for this discrepancy need to be clarified since resolution might yield a better insight into the general problems of utilizing iodinated hormones for investigating the binding and action of hormones at their receptors.

Iodine is principally and equally incorporated into the two tyrosyl residues of glucagon (Desbuquois, 1975). Iodination of tyrosyl residues produces two opposing effects: (i) an increase in the hydrophobicity of the phenoxy ring, and (ii) a reduction in the pK_a for hydroxyl group of tyrosine. A major effect due to iodination of peptide hormones can be expected if the iodinated tyrosyl residues are critically involved in the function of these molecules. We considered the possibility that these two effects resulting from iodine incorporation may alter the binding and action of the iodinated glucagon on the hepatic adenylate cyclase system. By studying the binding and action of iodoglucagon at a pH where the phenoxy group is not ionized, the effect of increased hydrophobicity can be examined. In this report, we provide evidence that ionization of the iodinated tyrosyl residues results in a marked decrease in the binding of glucagon, whereas the un-ionized species displays both an increased affinity for the glucagon receptor and increased potency in the activation of adenylate cyclase. Studies to be reported elsewhere[1] will deal with the binding kinetics of unlabeled native glucagon.

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