Direct Observation of the Phase Behavior of the Lipid Bilayers of Phage PM2 and the Intact Host Cells by ^1H-^{31}P Cross-Polarization NMR†

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ABSTRACT: A method for obtaining the ^{31}P NMR spectrum of a particular supramolecular structure in an intact biological system was developed by applying the ^1H-^{31}P cross-polarization technique to a lipid-containing bacteriophage, PM2, and its host bacterium, *Alteromonas espejiana*. It was shown that ^{31}P NMR spectra of nucleic acids and lipid bilayers can be obtained separately with short and long thermal contact times, respectively. The temperature dependence of the chemical shift anisotropy ($\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$) was examined for the separately obtained membrane spectra. Referring to the results of thermal analysis and ^{31}P NMR spectra of bilayers of the extracted phospholipids, the phase transition of the biomembrane was identified for the PM2 phage and the host cell. The dynamic state of the biomembrane of the intact bacterium was directly monitored in detail. The phase behavior of the PM2 lipid bilayer showed good agreement with the earlier report (Akutsu et al., 1980). It turned out that the phase behavior of the intact biomembrane is different from that of the bilayer of the extracted lipids for both PM2 and the host cell. Namely, the terminal temperatures of the phase transition of the host cell and PM2 membranes were lower and higher than those of the extracted phospholipids, respectively.

Many of the important reactions in a cell take place on biological membranes. Biomembranes are made up of lipids and proteins, comprising highly ordered supramolecular structures, which play key roles in the organization of the concerted activities in a biological system. Therefore, information on the biomembrane of an intact cell would be very important for investigation of in vivo systems. Solid-state ^{31}P NMR is one of the most powerful tools for studies on phosphorus-containing molecules undergoing limited motion, such as phospholipids in biomembranes (Burt, 1987). The conventional single-pulse technique, however, cannot be used for studies on complicated systems such as an intact cell, since the resonances of all phosphorus-containing molecules overlap one another in the spectrum because of the extraordinarily broad line width of molecules undergoing suppressed motion. To obtain meaningful information, the signal of interest should be observed separately. For this purpose, the ^1H-^{31}P cross-polarization technique has been shown to be promising (Akutsu, 1986; Nishimoto et al., 1987). Through the use of this technique, the ^{31}P NMR spectra of chromatins in intact chicken erythrocytes (Nishimoto et al., 1987) and nucleic acids in intact bacterial cells (Odahara et al., unpublished) have been selectively observed. ^{31}P and ^{13}C cross-polarization/magic angle spinning NMR were also applied to viruses by DiVerdi and Opella (1981) and Hemminga et al. (1981), respectively.

In this work, we further developed this method to obtain information on the biomembranes of intact biological systems. A lipid-containing bacteriophage, PM2, and its host cell, *Alteromonas espejiana*, were used for this purpose. *A. espejiana* is a marine bacterium, the optimal growth temperature of which is within the range 20–25 °C (Espejo & Canelo, 1968). Phage PM2 was extensively investigated by means of biochemical and biophysical methods (Mindich, 1978; Franklin et al., 1976; Franklin, 1974). It consists of four kinds of proteins (73.1% by dry weight), lipids (12.6%), and circular double-stranded DNA (14.3%) (Franklin, 1974). There are only two different phosphorus-containing molecular species, namely, phospholipids and DNA. Because of the simplicity of the components, the intact phage was investigated by conventional ^{31}P NMR, on which there was overlapping of the two powder patterns for phospholipids and DNA (Akutsu et al., 1980). In contrast, the host cell comprises a much more complicated system. It consists of many kinds of metabolite molecules and a variety of RNAs, in addition to thousands of proteins, lipids, and DNA. Therefore, phage PM2 and its host cell provide good model systems for a new method for investigating supramolecular systems in vivo.

Our results showed that the ^1H-^{31}P cross-polarization method can be used to obtain a membrane spectrum selectively, even in such a complicated system as a cell, and that such information is useful for investigation of the infection mechanism of a lipid-containing phage.

Materials and Methods

Purification of bacteriophage PM2 was carried out according to the modified procedure of Hinnen et al. (1974). All experiments were performed at 4 °C. Cell debris was removed from a lysate by continuous centrifugation at 13000 rpm (Tomy No. 15S) and the phages were precipitated by incubation of the lysate in the presence of 5% (w/v) poly(ethylene glycol) 6000 and 0.5 M NaCl for 48 h. The precipitate was collected by centrifugation at 9000 rpm (Tomy RPR-12) for 15 min. The pellet was resuspended in three volumes of buffer B1 (1 M NaCl, 10 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.2 at 25 °C) (Hinnen et al., 1974). After low-speed centrifugation, the suspension was layered on top of a four-step CsCl gradient (455.4, 386.6, 322.7, and 263.3 g of CsCl were added to 1 L of buffer B1). The phage was recovered at the interface of the third and fourth steps after centrifugation at 24000 rpm (Beckman SW28 rotor) for 110 min. The phage fraction was

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collected and its CsCl concentration was adjusted to 23.14% (w/w) by dialysis. Then it was centrifuged at 26000 rpm (Beckman type 40 rotor) for 20 h to obtain a linear gradient of CsCl. The collected phage fraction was dialyzed against a solution of buffer B1 containing 2 M NaCl (buffer B2) to remove CsCl. In order to avoid destruction of the phage particles and to suppress their rotational motion due to the high viscosity of both the solute and the solvent, the dialysis was carried out in the presence of sucrose, the concentration of which was changed stepwise, 10, 30, and 60% (w/w) (Akutsu et al., 1980). ^{31}P NMR spectra were measured with about 350 mg of the phage in the presence of 60% sucrose.

E. coli phage λ was prepared according to the method of Yamamoto and Alberts (1970). In order to obtain a high concentration by volume reduction and to suppress the rotational motion, the phage preparation for NMR measurements was dialyzed against λ buffer (10 mM Tris, 1 mM $MgCl_2$, 0.1 M NaCl, and 0.001 g of gelatin, pH 7.5) containing 30% sucrose. About 200 mg of the phage was used for the measurements. There was no difference in the spectra of the samples in the presence of 30% and 60% sucrose.

A. espejiana cells were prepared as follows. Three milliliters of an overnight culture of *A. espejiana* was added to 300 mL of Q medium [0.7 g of KCl, 1.5 g of $CaCl_2 \cdot 2H_2O$, 12 g of $MgSO_4 \cdot 7H_2O$, 26 g of NaCl, 5 g of yeast extract (DIFCO), and 10 g of Bacto Tryptone (DIFCO), in 1 L of deionized water, pH 7.4], which contained yeast extract and Bacto Tryptone instead of Bacto Nutrient Broth (Franklin et al., 1969). The cells were cultured at 25 °C to the middle of the logarithmic phase. Then the bacterial cells were immediately harvested at 4 °C by centrifugation at 10000 rpm (Tomy RPR-12) for 15 min and the pellet was put into an NMR tube of 10-mm diameter. A fresh culture was used for each NMR measurement.

Ribosomes of *A. espejiana* were purified as described by Cox et al. (1964). The pellet obtained on centrifugation at 60000 rpm (Beckman type 65 rotor) was suspended in 4 volumes of NTCM buffer (0.5 M NaCl, 10 mM $CaCl_2$, 20 mM $MgCl_2$, 6 mM mercaptoethanol, and 10 mM Tris-HCl, pH 7.2, at 25 °C). Either the suspension or a solution of it dialyzed against a solution of NTCM buffer containing 60% sucrose was used for the NMR measurements.

The total phospholipids of the PM2 phage and host cells were obtained according to the method of Bligh and Dyer (1959). They were washed with a 2 mM EDTA solution at pH 7 to remove divalent ions. Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were purified from the total phospholipid fraction of the host cells by a combination of silicic acid and DEAE-52-cellulose (Whatmann) column chromatographies. On silicic acid column chromatography, PE and PG were eluted with chloroform/methanol, 95/5 (v/v) and 80/20 (v/v), respectively. On DEAE-52-cellulose column chromatography, PE was eluted with chloroform/methanol, 8/2 (v/v), and PG with chloroform/methanol/ammonia water/ammonium acetate (160 mL/40 mL/4 mL/760 mg) (Law & Essen, 1969). For NMR measurements, 300 mg of the lipids was dispersed in 1 mL of buffer B1.

Cross-polarization and conventional single-pulse ^{31}P NMR spectra were obtained with a JEOL FX-100 NMR spectrometer equipped with a solid-state NMR system at 40.3 MHz. The field was locked on external deuterium oxide (D_2O). A probe head specially designed for high-power and variable-temperature measurements was used, in which the sample tube is held vertically. A 45° pulse and a 3.0- or 3.6-s relaxation delay were used for the single-pulse measurements. The same relaxation delay was used for the cross-polarization pulse sequence. The proton spins were irradiated during data acquisition. Temperature calibration for the thermal contact time and proton decoupling was carried out. The number of data points used for a spectral width of 50000 Hz was 4096. Phosphoric acid, 85%, was used as an external standard. ^{31}P high-resolution NMR spectra of intact *A. espejiana* cells were obtained with a Bruker WM360wb NMR spectrometer without proton irradiation. Thermograms of the phospholipid dispersions were obtained with a Privalov-type calorimeter, DASM-4. The scan rate was 0.25 K/min. The phospholipid concentrations were 10 and 5 mg/mL for the host cells and phage PM2, respectively.

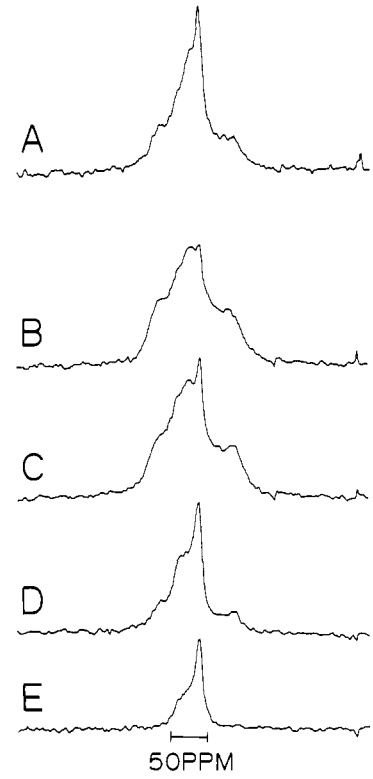

FIGURE 1: ^{31}P NMR spectra of intact bacteriophage PM2 in buffer B2 containing 60% sucrose at 5 °C. (A) Single-pulse spectrum with accumulation of 20000 free induction decays. (B, C, D, and E) Cross-polarization spectra with thermal contact times of 0.7, 1.0, 3.0, and 6.0 ms, respectively. For B, C, D, and E, 8000, 8000, 20000, and 28000 transients were accumulated, respectively. The 90° pulse width was 13.5 μs. A 3.6-s relaxation delay and an exponential window function with 100-Hz line broadening were used.

RESULTS

^1H-^{31}P Cross-Polarization Spectra of Bacteriophage PM2. Bacteriophage PM2 is a good model system for studying a membrane in the intact state because of its simple composition. Figure 1A shows a ^{31}P NMR spectrum of intact PM2 in buffer B2 containing 60% sucrose, measured in the conventional single-pulse mode, at 5 °C. All phosphorus-containing compounds in the phage particle contribute to this spectrum. This spectrum was interpreted in terms of asymmetric and axially symmetric powder patterns (Akutsu et al., 1980). The phage only contains two phosphorus-containing species, namely, phospholipids in its lipid bilayer and DNA packaged in its core. The asymmetric and axially symmetric powder patterns were assigned to the DNA and lipid bilayer, respectively. For an extensive investigation, however, it is desirable to obtain each signal separately. For this purpose, the cross-polarization technique was applied to this system. The efficiency of cross-polarization ($M_P(t)$) is a function of the spin–lattice

relaxation time of protons in the rotating frame ($T_{1\rho}$) and the cross-relaxation time (T_{HP}) between the protons and phosphorus, as shown in eq 1, where $\alpha = \gamma_P B_{1P}/\gamma_H B_{1H}$, $\epsilon = N_P/N_H$,

$$M_P(t) = K(1 + \epsilon\alpha^2 - T_{HP}/T_{1\rho})^{-1}[\exp(-t/T_{1\rho}) - \exp(-t(1 + \epsilon\alpha^2)/T_{HP})] \quad (1)$$

and $K = \alpha(\gamma_H/\gamma_P)M_{P0}$ (Mehring, 1983; Akutsu, 1986). Here t, γ, B, N, and M_{P0} represent the contact time, the gyromagnetic ratio, the amplitude of the rotating magnetic field, the number of spins, and the Zeeman magnetization of phosphorus spins, respectively. The efficiency is dominated by T_{HP} and $T_{1\rho}$ with short and long thermal contact times, respectively. These time constants are closely connected with the mode of molecular motion (Akutsu, 1986). Since phospholipids and DNA in the phage particle are undergoing quite different types of motions, the contact time dependence of the cross-polarization efficiency is expected to be different for them. Thus, we measured the cross-polarization ^{31}P NMR spectra of intact bacteriophage PM2 with a variety of contact times, which are shown in Figure 1B–E. The spectrum obtained with a contact time of 0.7 ms (Figure 1B) showed a typical asymmetric powder pattern, which corresponds to the broader component in the single-pulse spectrum. The principal values of its chemical shift tensor (σ_{11}, σ_{22}, and σ_{33}) were 70, 6, and −68 ppm. Its chemical shift anisotropy ($\Delta\sigma = \sigma_{33} - \sigma_{11}$) was −138 ppm. This is close to the value estimated from the overlapped spectrum (Akutsu et al., 1980). With a longer thermal contact time, a narrower component was obtained at the expense of the asymmetric powder pattern. Finally, this narrow component dominated the spectrum as a simple axially symmetric powder pattern with a chemical shift anisotropy ($\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$) of −44 ppm with a contact time of 6.0 ms (Figure 1E). This accounted for the narrow component in the single-pulse spectrum. These results support the explanation for the single-pulse spectrum in an earlier paper (Akutsu et al., 1980). In order to confirm the assignments, the cross-polarization characteristics of DNA in phage particles and phospholipid bilayers were examined.

The Efficiency of the Cross-Polarization Studied for Model Liposomes and the λ Phage. Phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) purified from the host cells were mixed at the ratio of 64/27 (w/w), which reflects the phospholipid composition in PM2 (Mindich, 1978). The cross-polarization spectrum of liposomes of this lipid mixture was measured with a variety of contact times at 5 °C. They showed axially symmetric powder patterns, one of which is presented in the insert in Figure 2A. The intensity minimum in the spectrum can be explained by weak dipolar interactions at the magic angle orientation (Frye et al., 1985). The minimum disappeared with longer contact times because of the lateral diffusion and/or tumbling motion of the vesicles. The integrated intensity of the spectrum is plotted as a function of the contact time in Figure 2A. T_{HP} and $T_{1\rho}$ were obtained by nonlinear least-squares fitting using eq 1, which gave 0.8 and 25.1 ms, respectively. The best-fit curve is shown by the solid line. The λ phage was used as a model of DNA packaged in a virus particle, since DNA is the single source of phosphorus in this phage. The cross-polarization spectrum of the intact λ phage is given in the insert in Figure 2B, which shows an asymmetric powder pattern. Its integrated intensity is plotted as a function of the contact time in Figure 2B. T_{HP} and $T_{1\rho}$ were obtained in a way similar to those for phospholipids and were 0.5 and 3.1 ms, respectively.

From both the type of powder pattern and the cross-polarization characteristics, we can definitely assign the asymmetric and axially symmetric powder patterns in Figure 1 to DNA and the phospholipid bilayer, respectively. On the basis of the results in Figure 2, however, a more significant contribution of the DNA signal to the spectrum with a contact time of 6.0 ms would be expected, while the contribution can hardly be seen in Figure 1E. This suggests that the real T_{HP} and $T_{1\rho}$ of DNA and the phospholipid bilayer in PM2 may be a little different from the values obtained from Figure 2. We can now conclude that the spectra of lipid bilayers and DNA can be obtained separately for such a simple system as the PM2 phage by choosing the optimum contact time.

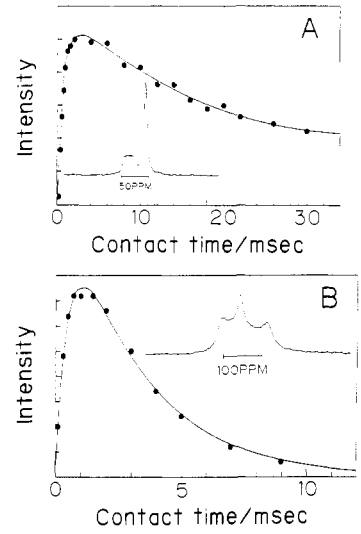

FIGURE 2: Contact time dependence of the integrated intensity of the cross-polarization ^{31}P NMR spectra at 4 °C. (A) Liposomes of the semi PM2 total phospholipid, which is a mixture of PE and PG from *A. espejiana* in the same ratio as for the lipid composition of the PM2 phage. (B) Intact λ phage in λ buffer with 30% sucrose. The solid curves were obtained by nonlinear least-squares fitting. The spectra with contact times of 6.0 ms (A) and 0.7 ms (B) are presented in the inserts.

^{1}H–^{31}P Cross-Polarization Spectra of Intact Cells of A. espejiana. Application of the cross-polarization technique was extended to a more complicated system, a bacterial cell, to check the general feasibility of this method. Figure 3A is the ^{31}P NMR spectrum of *A. espejiana* cells at 4 °C, measured in the conventional single-pulse mode, which includes the contributions from all phosphorus-containing molecules in a cell. Phosphorus nuclei are located in DNA, RNA, phospholipids, and many metabolites, such as ATP and sugar phosphate, in a bacterial cell. The sharp and symmetrical peak at the center comprises signals originating from small soluble molecules. Since an exponential window function with a 100-Hz broadening factor was used, the signals were not resolved. Actually, well-resolved signals were obtained in a ^{31}P high-resolution NMR spectrum (Seelig, 1986), as can be seen in Figure 3B. The broad components in Figure 3A can be attributed to supramolecular systems undergoing limited motion, such as phospholipids in the biomembrane, DNA in the chromosome complex, RNA in the ribosomes, and so on. The cross-polarization pulse technique is expected to get rid of the contributions from at least small molecules. Parts A, B, C, and D of Figure 4 show the cross-polarization ^{31}P NMR spectra of intact *A. espejiana* cells obtained with contact times of 0.7, 3.0, 5.0, and 7.0 ms, respectively. In all of these cross-polarization spectra, the sharp peak at the center was hardly seen, as expected. The pattern of the cross-polarization spectrum changed with the contact time, as in the case of PM2. In spite of the complexity of the system, the observed spectrum was rather simple. The cross-polarization spectrum with a

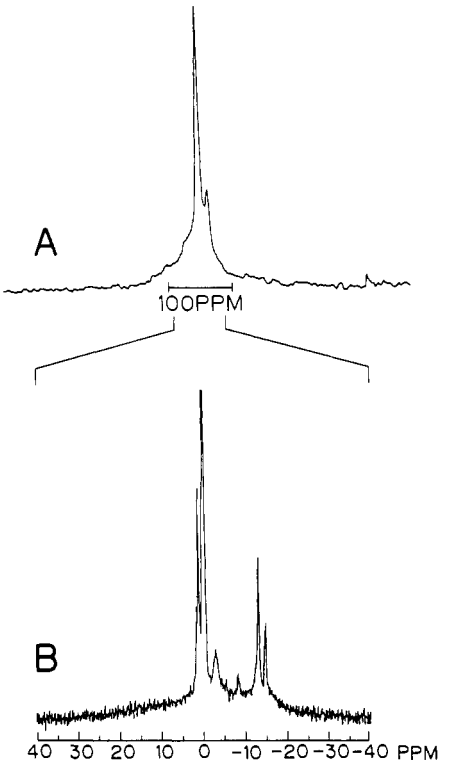

FIGURE 3: ^{31}P NMR spectra of intact *A. espejiana* cells measured in the single-pulse mode at 4 °C. (A) Single-pulse broad-line spectrum at 40.3 MHz with proton-noise decoupling and a 3-s relaxation delay: 3000 transients were accumulated and 100-Hz line broadening was used. (B) High-resolution spectrum with a 0.3-s relaxation delay and 3-Hz line broadening: 45 000 transients were accumulated.

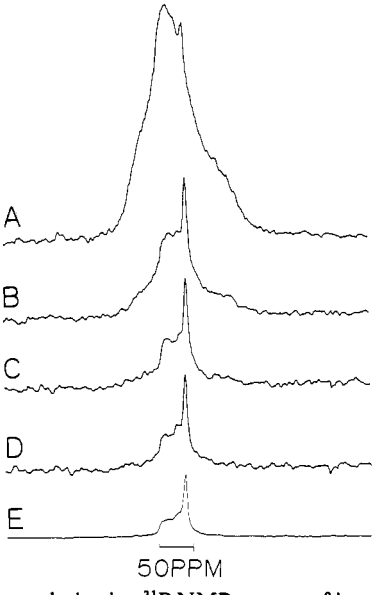

FIGURE 4: Cross-polarization ^{31}P NMR spectra of intact *A. espejiana* cells at 4 °C. A, B, C, and D were measured with contact times of 0.7, 3.0, 5.0, and 7.0 ms, respectively, with the accumulation of 3000 free induction decays. E was measured with a contact time of 7.0 ms with the accumulation of 12 000 scans. The 90° pulse width was 10.3 μs. A 3-s relaxation delay and 100-Hz line broadening were used.

thermal contact time of 0.7 ms (Figure 4A) showed an asymmetric powder pattern. This spectrum corresponds to the broadest component in the single-pulse spectrum and its chemical shift anisotropy is about −160 ppm. In spite of its large anisotropy, the principal values of the chemical shift tensor are not evident, suggesting either a broad distribution of the motions or the existence of slow motions at about 10^4

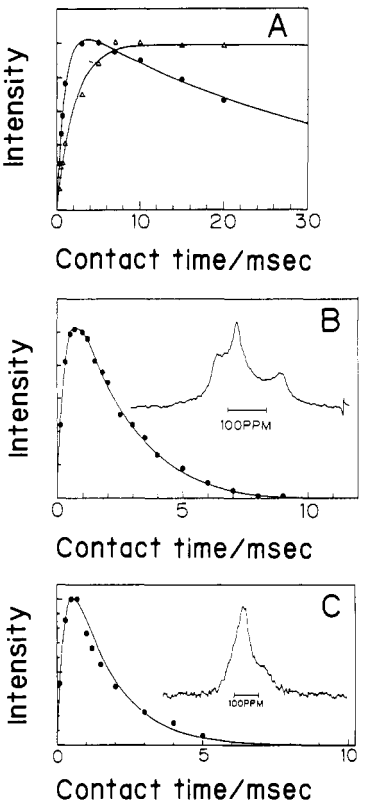

FIGURE 5: Contact time dependence of the integrated intensity of the cross-polarization spectra. (A) Liposomes of total phospholipids of *A. espejiana* at 4 °C (●) and 30 °C (△). The 90° pulse width was 10.2 μs. (B and C) Purified ribosomes of *A. espejiana*, at 4 °C, suspended in about 5 volumes of NTCM buffer and then dialyzed against NTCM buffer with 60% sucrose (B) and suspended in four volumes of NTCM buffer (C). The 90° pulse width was 10.3 μs. Relaxation delays of 3 and 4 s were used for liposomes and ribosomes, respectively. The spectra of ribosomes with a contact time of 0.7 ms are given in the inserts.

s^{-1} (Campbell et al., 1979). The spectrum with a thermal contact time of 7.0 ms gives rise to a pure axially symmetric powder pattern, as in the case of PM2. The chemical shift anisotropy was −44.7 ppm. The spectra observed with contact times of 3.0 and 5.0 ms can be interpreted by overlapping of the powder patterns with contact times of 0.7 and 7.0 ms at a different ratios. These results show that the weak sharp peak on the right in the single-pulse spectrum (Figure 3A) represents overlapping of the perpendicular component of the axially symmetric powder pattern and signals of soluble molecules.

Comparison of the Cross-Polarization Efficiency between Liposomes and Ribosomes. The major supramolecular structures containing phosphorus in a bacterial cell are nucleic acids and the biomembrane. Of the former, ribosomal RNA and DNA are known to be the most and second most major species (Watson, 1975). To identify the origins of the two powder patterns, ^{31}P cross-polarization spectra were examined for purified ribosomes and liposomes of the total phospholipid fraction extracted from the cells. The latter again showed an axially symmetric powder pattern. The ribosomes in NTCM buffer containing 60% sucrose gave rise to an asymmetric powder pattern with principal values of the chemical shift tensor of 88, 17, and −117 ppm, as shown in the insert in Figure 5B. The spectral intensity is plotted as a function of the contact time in Figure 5. Nonlinear least-squares fitting was carried out by using eq 1. Solid lines represent best-fitting curves. The obtained T_{HP} and $T_{1\rho}$ of the phospholipid bilayer were 0.9 and 38.2 ms at 4 °C and 2.1 and 1300 ms at 30 °C, respectively. As can be seen later (Figure 6A), the bilayer

of the total phospholipid fraction is in a broad phase transition at 4 °C and in the liquid-crystalline state at 30 °C. The $T_{1\rho}$ of the protons became longer with an increase in the motion of the phospholipids, suggesting that the dominant correlation time is in the fast motional regime. The maximum intensity was obtained with contact times of 3.0 and 7.0 ms at 4 and 30 °C, respectively. Similar results were reported for synthetic phosphatidylcholine bilayers (Akutsu, 1986). On the other hand, the maximum intensity of the cross-polarization spectra of the purified ribosomes in NTCM buffer containing 60% sucrose was observed with a contact time of 0.7 ms and the intensity decreased to one-twentieth the maximum with a contact time of 7.0 ms. T_{HP} and $T_{1\rho}$ obtained by nonlinear least-squares fitting were 0.3 and 2.3 ms, respectively. In order to imitate the situation in the cell, purified ribosomes suspended in 4 volumes of NTCM buffer were also examined at 4 °C. The results are shown in Figure 5C. The spectrum is similar to the cross-polarization spectrum of the intact host cells with a thermal contact time of 0.7 ms. The chemical shift anisotropy was −170 ppm. The maximum intensity was obtained with a thermal contact time of 0.6 ms. While the contact time dependence of the ribosomes was similar to that of DNA, the contact time for the intensity maximum was shorter than that in the case of DNA because of the shorter T_{HP} and $T_{1\rho}$.

These results allow us to assign the spectra with contact times of 0.7 and 7.0 ms to the nucleic acids and biomembrane of *A. espejiana* cells, respectively. For the former, the contribution of ribosomal RNA should be dominant because of its major presence. Actually, the intensity maximum of the cross-polarization spectrum of the host cells was observed with a contact time of 0.7 ms, which is in good agreement with that at the maximum for the purified ribosomes. This also supports the major contribution of the ribosomes. The contribution of DNA is expected to be one-sixth, according to the composition in *E. coli* (Watson, 1975). Therefore, it cannot be ignored. This complexity might be the reason for the rather featureless powder pattern of Figure 4A.

Although the supramolecular systems of nucleic acids can be observed in vivo by means of the cross-polarization method, it turned out to be difficult to extract more specific information on the chromosome complex, ribosomes, and so on unless one component was overwhelmingly dominant. In contrast, the information on the biomembrane is clear regardless of the complexity of the biosystems. We took advantage of this in exploring the dynamic nature of the biomembranes of phage PM2 and the host cell.

Phase Transition of the Phospholipid Bilayers in the Intact Biomembranes of PM2 and A. espejiana. In order to examine the correlation between the chemical shift anisotropy and phase behavior, the temperature dependence of the chemical shift anisotropy and the thermograms of the bilayers of the total phospholipid fraction extracted from *A. espejiana* cells and the PM2 phage were examined. The results are presented in Figure 6. Figure 6A shows the thermogram for the *A. espejiana* total phospholipid bilayer. The phase transition started from below 5 °C and finished at 24.5 °C. This asymmetric profile of the transition is characteristic of a PE-rich lipid bilayer (Jackson & Sturtevant, 1977). ^{31}P chemical shift anisotropy of the same phospholipid bilayer is plotted as a function of temperature in Figure 6B. The absolute value of the ^{31}P chemical shift anisotropy decreased gradually from about 0 to about 23 °C, which is in good agreement with the phase-transition range in the thermogram. The results show that the *A. espejiana* total phospholipid bilayer is in the liquid-crystalline state above 24.5 °C and in the process of a

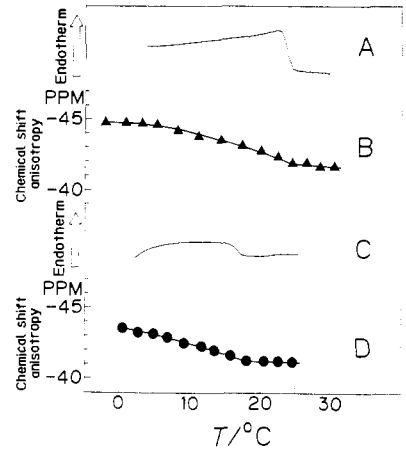

FIGURE 6: Thermograms (A and C) and temperature dependence of the ^{31}P chemical shift anisotropy ($\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$) (B and D) of total phospholipid liposomes of the PM2 phage and *A. espejiana*. (A and B) *A. espejiana* total phospholipids. (C) PM2 total phospholipids and (D) semi PM2 total phospholipids (see the legend to Figure 2).

broad phase transition in the range from 0 to 24.5 °C, where the liquid-crystalline and gel states coexist.

The thermogram of liposomes of the total phospholipid fraction extracted from the PM2 phage is presented in Figure 6C. The thermogram indicates that the phase transition started from below 5 °C and finished at about 17 °C. Thus, it can be said that the PM2 total phospholipid bilayer is in the liquid-crystalline state at above 17 °C and in the process of a broad transition below 17 °C. The different profile of this thermogram from that of the *A. espejiana* total phospholipid fraction can be attributed to the low phosphatidylethanolamine fraction (27%) in PM2. Since the amount of total phospholipids of the PM2 phage was not enough for measurement of a ^{31}P NMR spectrum, the temperature dependence of the ^{31}P chemical shift anisotropy was examined for a semi PM2 total phospholipid bilayer (a mixture of PE and PG from the host cells). The results are shown in Figure 6D. The absolute value of the ^{31}P chemical shift anisotropy decreased gradually up to about 18 °C and then plateaued at above that temperature. This also shows a good correlation with the results of thermal analysis. The reflective point represents the end of the phase transition. This coincidence shows in turn that the semi PM2 total phospholipid bilayer well reflects the nature of the genuine one.

Since the correlation between the phase behavior and ^{31}P chemical shift anisotropy had been well substantiated, *A. espejiana* and the PM2 phage were examined. Cross-polarization ^{31}P NMR spectra of intact *A. espejiana* cells with a contact time of 7.0 ms were obtained at several temperatures. Every measurement was performed with a fresh culture. All the spectra exhibited axially symmetric powder patterns. The chemical shift anisotropy of the spectrum is plotted as a function of the temperature in Figure 7A. The chemical shift anisotropy was −45 ppm at 0 °C and its absolute value decreased with an increase in temperature from 4 to 30 °C. However, the slope of the plot above 11 °C is different from that in the range from 0 °C to about 11 °C. The slope in the lower temperature range is steeper, giving a reflective point at about 11 °C. This bacterium is known to die above 30 °C (Espejo & Canelo, 1968), which is reflected in the chemical shift anisotropy at the highest temperature. The ^{31}P chemical shift anisotropy of the membrane of the intact PM2 phage is plotted as a function of temperature in Figure 7B. All the cross-polarization spectra of intact PM2 with a contact time of 6.0 ms also showed axially symmetric powder patterns. The

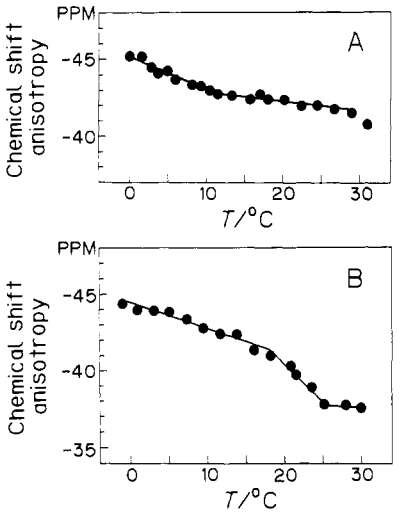

FIGURE 7: Temperature dependence of the ^{31}P chemical shift anisotropy of biomembranes in vivo. (A) Intact *A. espejiana* cells. A fresh culture was used for each measurement. (B) The intact PM2 phage in buffer B2 with 60% sucrose. The Hartmann–Hahn condition was redetermined for every measurement.

^{31}P chemical shift anisotropy was −44 ppm at about 0 °C. Its absolute value decreases gradually up to about 17 °C, followed by a great decrease, and plateaus at about 25 °C.

Discussion

^1H–^{31}P cross-polarization was shown to be useful for the investigation of lipid bilayers (Akutsu & Kyogoku, 1984; Akutsu, 1986) and the chromatin in intact chicken erythrocytes (Nishimoto et al., 1987). Both of these systems are, however, very extreme cases. In the former, the origin of the spectrum is self-evident, and in the latter, the contributions of RNA and the lipid bilayer are negligible or very small. It was shown in this work that the availability of this method is more general. The selectivity of the information is especially high for biomembranes. A pure lipid bilayer spectrum could be obtained for intact bacterial cells. In the case of nucleic acids, the assignments are not straightforward. Nevertheless, the major contribution should come from DNA and/or ribosomes. The contact time dependence of the spectral intensity can give an idea of which one is dominant. A chemical composition surely helps the analysis.

The separation of the spectra of nucleic acids and a lipid bilayer is not accidental. The lipid bilayer has much more freedom of motion because it is composed of small lipid molecules that undergo rapid axially symmetric motion (Kohler & Klein, 1977; Seelig, 1978). Double-stranded DNA in a chromosome complex and RNA in ribosomes are much more rigid. Thus, the cross-relaxation time, T_{HP}, is longer for lipid bilayers than for nucleic acids. The correlation time of the proton motion in lipid bilayers in the gel and liquid-crystalline states falls in the fast motional regime in the rotating frame (Akutsu, 1986), while that of chromatin DNA falls in the slow motional regime (Nishimoto et al., 1987). Since $T_{1\rho}$ of nucleic acids is close to its minimum (Akutsu et al., unpublished), it is usually shorter than $T_{1\rho}$ of lipid bilayers. The longer T_{HP} and $T_{1\rho}$ of lipid bilayers, as compared to those of nucleic acids, permit the selective observation of their spectra. However, a longer cross-correlation time leads to a lower signal-to-noise ratio in the spectrum of a lipid bilayer. Another interesting point is that the abnormal intensity minimum observed for the extracted lipids, as shown in the insert in Figure 2A, was not observed for the intact biological system. This suggests that the mode of fluctuation of the polar groups is different for simple lipid bilayers and intact biomembranes (Frye et al., 1985).

The application of this method to the PM2 phage and its host cell was satisfactory. The results of thermal analyses and phosphorus chemical shift anisotropy measurements of extracted lipids showed a good correlation. These results provided a firm base for analysis of the intact biological system. Although the phase behaviors of the lipids extracted from *A. espejiana* cells and the PM2 phage were investigated by ESR (Tsukagoshi et al.,1976b) and NMR (Akutsu et al., 1980), respectively, no clear conclusion was obtained. The present work clearly showed that the pure extracted lipid bilayers exhibit broad phase transitions up to 17 and 24.5 °C for the PM2 phage and the host cells, respectively. They are in the liquid-crystalline state above these temperatures. The difference between the two temperatures can be attributed to the difference in the phospholipid composition, since their fatty acid compositions are expected to be similar (Camerini-Otero & Franklin, 1972). The phospholipid composition is 27% phosphatidylethanolamine (PE) and 64% phosphatidylglycerol (PG) for the PM2 phage (Braunstein & Franklin, 1971; Tsukagoshi et al., 1976a) and 72% PE and 23% PG for the host cells (Diedrich & Cota-Robles, 1974). Since the phase-transition temperature of phosphatidylglycerol bilayers is generally lower than that in the case of phosphatidylethanolamine, the difference in the phase transition temperature mentioned above is reasonable.

The phase transition of the *A. espejiana* membrane took place between lower than 4 and 11 °C. The phase behavior of the purified membranes of this bacterium was investigated by ESR, using a spin label reagent (Tsukagoshi et al., 1976b). The phase transition temperature determined from the reflection point was 12 °C. Our result agrees with this, provided that this is the end of the phase transition. However, the end point of the phase transition is different from that in the case of extracted lipid bilayers by about 13 °C. This is rather surprising because many reports have shown that the phase behavior is more or less similar for biomembranes and extracted lipid bilayers. However, this is reasonable from the biological point of view in this case, since the biomembrane should be in the liquid-crystalline state at a physiological temperature (20–25 °C). The difference in the phase behavior should be due to the organization of biomembranes. It is noteworthy that the phase-transition temperature of intact cells of *Acholeplasma laidlawii* is lower than even that of isolated membranes (Cameron et al., 1983). Although the exact reason for the change in the phase behavior cannot be given at this stage, this phenomenon seems to have general significance.

The change in the chemical shift anisotropy of the PM2 phage was greater than those in the cases of the extracted phospholipids and the intact host cells, resulting in a smaller absolute value at a higher temperature. This suggests that some motions causing averaging of the chemical shift tensor are involved. Since the diameter of this virus is about 60 nm (Silbert et al., 1969), lateral diffusion and/or rotation of the particle can cause the averaging (Burnell et al., 1980). No averaging was observed up to 32 °C for the same virus particle in the presence of 60% sucrose (Akutsu et al., 1980), suggesting that the contribution of the lateral diffusion is negligible in this temperature range. Thus, the smaller chemical shift anisotropy value obtained in this work may be attributed to the rotation of the particle due to its lower concentration, which resulted in a lower viscosity of the solution. A part of the gradual change in Figure 7 can be attributed to the increase

in the rotational motion with the increase in temperature. The change between 17 and 25 °C, however, cannot be explained by such a motion. Consequently, it can be ascribed to the phase transition of the lipid bilayer of the PM2 phage. This coincides with the conclusion drawn in the previous work (Akutsu et al., 1980). The temperature range of the phase transition of the PM2 membrane is much narrower than that of the extracted lipids. Furthermore, the temperature at the end of the phase transition of PM2 is higher than that of the extracted lipids by 8 °C, in contrast to the case of *A. espejiana* cells. Since the structure of this virus has been well established, we may discuss these features in more detail. In the PM2 phage, phospholipids are located in an asymmetrical manner; namely, most of the phosphatidylethanolamine is located inside and most of the phosphatidylglycerol outside of the nucleocapsid membrane (Schäfer & Franklin, 1975). The nucleocapsid was covered by coat proteins (protein II), which are basic (pI 9–12.3; Schäfer et al., 1974). Furthermore, half of the volume of the nucleocapsid membrane is occupied by the capsid proteins (protein III) (Schneider et al., 1978). The high content of phosphatidylethanolamine in the inner leaflet of the bilayer would raise the phase-transition temperature of the inner leaflet. Furthermore, the interaction between phosphatidylglycerol and basic coat proteins would also raise the phase transition temperature of the outer leaflet. Protein–protein interactions between the capsid proteins and between the capsid proteins and coat proteins may affect the dynamic state of the lipid bilayer as well. These interactions would introduce more cooperativity in the dynamic structure of the lipid bilayer, which would result in a sharper phase transition. Thus, the increase in the phase transition temperature and the sharper transition in the case of the PM2 membrane are consistent with the proposed structural model of the phage.

In a previous study (Akutsu et al., 1980), the early event of the infection of the host cell by the PM2 phage was examined as a function of temperature. Enhancement of the infectivity was observed in the temperature region of 15–23 °C. It was concluded that the change in the dynamic state of the phage membrane might be involved in this early event. The results of this work support this conclusion. In the first place, it was confirmed that the phase transition of the cell membrane of the host bacterium takes place in quite a different temperature range from the above one. Thus, the alteration in the dynamic state of the host cell biomembrane cannot be directly correlated with the infectivity of PM2. Second, it was confirmed that the phase transition of the phage membrane occurs in the same temperature range as shown above. Consequently, it is reasonable to regard the dynamic structure of the phage particle as one of the main factors for the alteration in the phage infectivity.

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