A Spin-Label Study on Fusion of Red Blood Cells Induced by Hemagglutinating Virus of Japan†

Toyozo Maeda, Akira Asano, Kazunori Ohki, Yoshio Okada, and Shun-ichi Ohnishi*

ABSTRACT: Fusion of red blood cells (RBC) induced by hemagglutinating virus of Japan (HVJ) has been studied using a phosphatidylcholine spin label. The spin label was readily incorporated and diffused into the lipid bilayer portion of the viral envelope. The exchange broadening in the electron spin resonance (ESR) spectrum of densely labeled virus disappeared rapidly when the virus was mixed with RBC at 37°. The spectrum gradually approached that of the host cell spin labeled with the phosphatidylcholine label. The results directly indicate transfer and intermixing of phospholipid molecules between the viral envelope and RBC membrane. The transfer reaction was strongly dependent on temperature. No transfer was observed at lower temperatures where the virus adsorbed to the cell and caused aggregation but no hemolysis and fusion. The transfer rate remained negligibly small until 19° and increased rapidly between 25 and 30°. The virus-induced hemolysis showed similar temperature dependence. The transfer rate was greatly reduced under inhibitory conditions of fusion: glutaraldehyde treatment of RBC, trypsin treatment of HVJ, or the presence of concanavalin A. Only slight transfer was observed from fusion-inactive influenza virus to RBC. The transfer was greatly enhanced by the help of HVJ. The close parallelism suggests that the transfer and intermixing are necessary steps to the cell fusion. The transfer rate was dependent on fluidity of the host cell membrane and independent of the viral dose. The virus-induced transfer of phospholipid molecules between RBC's was also detected by the spin label. Its temperature dependence was quite similar to that for the virus-to-cell transfer. The intercellular transfer was nearly proportional to the viral dose.

Membrane fusion constitutes a critical step in many significant cellular processes such as pinocytosis, phagocytosis, release of secretory products from cells, etc. (see, for example, Poste and Allison, 1973). Virus-induced fusion of mammalian cells provides a suitable model system for membrane fusion and there have been published a number of investigations on the system. Morphological studies have disclosed some key steps leading to the fusion. The envelope of hemagglutinating virus of Japan (HVJ[1]) fused to red blood cell (RBC) membrane (Howe and Morgan, 1969). The envelope fusion caused aggregation of glycoproteins on RBC (Bächi et al., 1973). The envelope fusion with phospholipid model membranes containing gangliosides was also observed to occur (Haywood, 1974).

The spin-label technique can be successfully used in the study of molecular mechanism of cell fusion. The method is to observe disappearance of exchange broadening in the electron spin resonance (ESR) spectrum when densely labeled membranes are mixed with unlabeled membranes. Its potential usefulness has been demonstrated in our previous study on phospholipid vesicles (Maeda and Ohnishi, 1974). Phospholipid molecules were rapidly transferred between phospholipid vesicles at the instant of membrane contact through vesicular collision. Fusion of vesicles was suggested to occur when larger quantities of phospholipid molecules were transferred in larger area of membranes. In the present investigation, we have used spin-labeled HVJ to obtain some molecular insight into the virus-induced fusion of RBC. The results have directly shown that the transfer and intermixing of phospholipids occurred between the viral envelope and RBC membrane. The virus-induced transfer of phospholipids between RBC membranes has also been observed. These results are discussed in relation to the fusion mechanism.

Materials and Methods

Virus. HVJ, z strain, was used throughout. The virus was grown in the allantoic cavity of 10-day-old eggs for 3 days at 37°. HVJ was partially purified from the infected fluid by differential centrifugation (700*g* for 10 min and 34,000*g* for 30 min), suspended in Ca^{2+}-free BSS solution (NaCl (140 m*M*), KCl (54 m*M*), Na_2HPO_4 (3.4 m*M*), KH_2PO_4 (4.4 m*M*), and Tris-HCl (10 m*M*), pH 7.6), and centrifuged at 700*g* for 10 min. Final supernatant solution was stored at −20°. In one experiment, HVJ was further purified by density gradient centrifugation to remove possible contamination of microsome which originated from the host cell. However, the purification did not affect the results described later. Trypsin treatment of HVJ was carried out at 35° for 20 min in a solution of 20 μg/ml of trypsin (bovine, grade 3, Miles-Seravac) and 10 m*M* phosphate buffer (pH 7.2). Influenza virus A_0PR8 was kindly supplied by Dr. T. Iwasaki, Institute for Virus Research, Kyoto University, as the allantoic fluid prepared from egg. The virus was purified by differential centrifugation (6000*g* for 10 min, 40,000*g* for 50 min, and 6000*g* for 10 min). The final supernatant solution was used for spin labeling. Virus concentration was expressed as hemagglutinating units (HAU) per

† From the Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan, the Institute for Protein Research, Osaka University, Osaka 565, Japan, and the Research Institute for Microbial Diseases, Osaka University, Osaka 565, Japan. *Received February 10, 1975.*

[1] Abbreviations used are: HVJ, the hemagglutinating virus of Japan; synonym, Sendai virus; RBC, red blood cell; HAU, the hemagglutinating unit; PC*, spin-labeled phosphatidylcholine where the β-fatty acid chain was replaced with the 4′,4′-dimethyloxazolidinyl-*N*-oxy derivative of 12-ketostearic acid; HVJ* and RBC*, HVJ and RBC spin labeled with PC*; BSS solution, NaCl-KCl-Na_2HPO_4-KH_2PO_4-Tris-HCl.

milliliter of solution or by the protein concentration. Titration of hemagglutinating activity was done by Salk's pattern method (see Okada and Murayama, 1968). Protein concentration was determined by the method of Lowry et al. (1951). Unless otherwise stated, experiments were carried out in Ca^{2+}-free BSS solution.

Red Blood Cell. Human RBC were obtained from the blood bank and used within about 2 weeks from drawing. Fish RBC were prepared from dace (*Tribolodon hakonesis*) or from crucian carp (*Carassius carassius*) fished in a local stream. RBC were rendered free of buffy coat by at least three washings in BSS solution. Treatment of RBC with glutaraldehyde (0.1%) was performed at 37° for 20 min. Glutaraldehyde (70%) was obtained from Ladd Research Industries.

Preparation of Spin-Labeled Virus and RBC. Spin-labeled phosphatidylcholine (PC*) was prepared by the reaction of egg lysolecithin and the anhydride of 12-nitroxide stearic acid according to Hubbell and McConnell (1971). The product gave a single spot on the silica gel thin-layer chromatogram. PC* (2 mg) was suspended in 1 ml of BSS, sonicated in an ice bath for 10 min under a nitrogen stream with a 10-kHz tip-type sonicator (Kaijo Denki), and centrifuged at 4° for 60 min at 100,000*g* to remove the small amount of undispersed lipid. The virus (1 mg of protein per ml) was incubated with the PC* dispersion (~1 m*M*) at 30° for 2 hr. After the incubation, the labeled virus was washed twice by centrifugation at 26,000*g* for 30 min at 4° and suspended in BSS. In one experiment, HVJ* was further purified by centrifugation through a stepwise density gradient consisting of 0.2 ml of 40% sucrose and 4 ml of 20% sucrose (60,000*g* for 30 min at 4°). However, the purification did not alter the experimental results described later. HVJ was also spin labeled by a different method using Nonidet P 40 (Hosaka and Shimizu, 1972). PC* (83 μl) (~2 m*M*) and 145 μl of Nonidet P 40 (final concentration 0.2%) were added to 0.5 ml of HVJ (2.5 mg of protein per ml). After standing for 15 min at room temperature, the mixture was applied to a Sephadex G-200 column (0.7 × 40 cm) at 4°. The first peak of the two eluted peaks was collected and concentrated by centrifugation for ESR measurements. Nonidet P 40 was a gift of Dr. K. Tamura of the Institute for Virus Research, Kyoto University. The content of PC* incorporated in HVJ* was estimated after extraction of total lipid from HVJ* by the method of Bligh and Dyer (1959). Concentration of the label was determined from the ESR signal intensity and the phospholipid content was obtained by the method of Bartlett (1959).

Human RBC was spin labeled by the incubation of 0.1 ml of 100% (v/v) RBC with 0.1 ml of PC* (2 m*M*) for 1 hr at 40°, followed by removal of the unincorporated PC*. Densely labeled RBC was prepared by incubation for a longer time (3 hr). The fish RBC was similarly spin labeled by the incubation of 0.1 ml of 100% RBC with 0.05 ml of PC* at 40° for 25 min.

Assay of Hemolysis and Fusion and ESR Measurement. HVJ* (0.1 ml) (2–4 × 10^4 HAU/ml) was added to 1 ml of 10% RBC and the mixture was diluted to the final volume of 4 ml with BSS solution. In one experiment, 80 μg of concanavalin A (Boehringer) was added to the mixture after 1 min to inhibit fusion. The mixture was left for 15 min at 0°. A part of the mixture was then centrifuged down. The packed cells were taken in a quartz capillary tube and incubated at 37° for varying times, and the ESR spectrum was measured. The residual mixture was used to measure hemolysis and fusion. An aliquot of the mixture was incubated at 37° for 15 min and photographed by a light microscope to measure fusion. The hemolysis activity was determined spectrophotometrically at 540 mμ in the oxy or cyanomet form of hemoglobin after removal of nonlysed RBC by centrifugation. The total hemoglobin content was obtained by complete lysis of RBC with 0.2% Triton X-100.

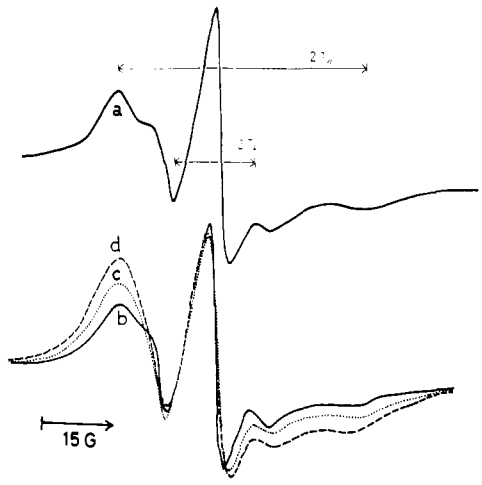

FIGURE 1: ESR spectrum of HVJ spin labeled under various conditions: (a) HVJ (1.5 mg of protein/ml) was incubated with PC* (0.5 m*M*) at 30° for 1 hr; (b, c, and d) HVJ (10 mg of protein/ml) was incubated with PC* (2 m*M*) at 30° for 20 min, 7 hr, and 12 hr, respectively. Spectra were measured at 21°. The parallel and perpendicular principal values, $T_{\parallel}$ and $T_{\perp}$, were obtained from the indicated splittings. The order parameter was calculated by $(T_{\parallel} - T_{\perp})/27$. The membranes are considered to be more fluid when the order parameter was smaller or the overall splitting ($2T_{\parallel}$) was smaller.

ESR spectra were measured with a commercial X-band spectrometer (JEOLCO Model ME-2X) at about 23°.

Results

Incorporation of PC into the HVJ Envelope.* PC* was readily incorporated into the HVJ envelope by incubation of the virus with sonicated PC* dispersion. The ESR spectrum of the spin-labeled virus showed the characteristics of the labels undergoing rapid anisotropic motion (see Figure 1a). The parallel and perpendicular principal values of the hyperfine tensor were 27.2 and 6.5 G at 21°, respectively. The order parameter was calculated to be 0.83 from the values, indicating that the envelope included a rather rigid bilayer membrane. The spectrum was dependent on temperature. The order parameter decreased with the rise of temperature: 0.67 at 30°, 0.62 at 37°, and 0.47 at 45°. As the incubation time increased, the amount of incorporated label increased and the spectrum of HVJ* became gradually broadened by the spin–spin exchange interaction (Figure 1b–d). For example, the ratio of incorporated PC* to viral phospholipid reached as high as 0.25 after 12-hr incubation at 30°. When more concentrated PC* was used, the amount of incorporation became larger. HVJ* prepared by another method, i.e. by treatment with Nonidet P 40 in the presence of PC*, followed by removal of the detergent through column, showed essentially the same ESR spectrum. Phosphorus analysis indicated that almost all the labels were incorporated. These results show that PC* was incorporated and diffused rapidly in the lipid bilayer portion of HVJ.

The hemagglutinating, hemolytic, and fusion activities of the virus were not affected by the spin labeling. For example, HVJ* containing 8.6% of PC* exhibited almost the

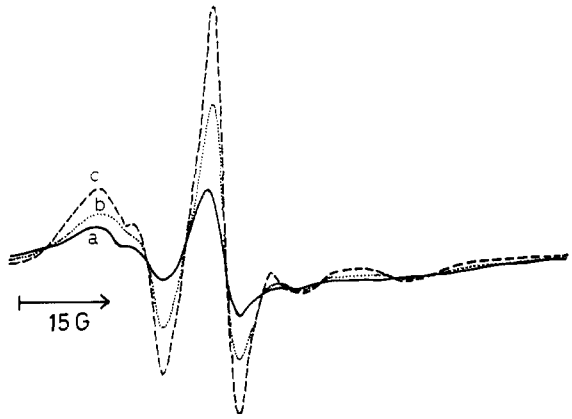

FIGURE 2: Change in ESR spectrum of HVJ* when incubated with RBC at 37° for (a) 0 min, (b) 7.5 min, and (c) 60 min. HVJ* and RBC were mixed at 0° (final concentration, 2000 HAU/ml and 2.5%, respectively) and the resulting pellet of RBC aggregate was taken in a quartz capillary tube and incubated at 37°. The ESR spectrum was measured at 21°.

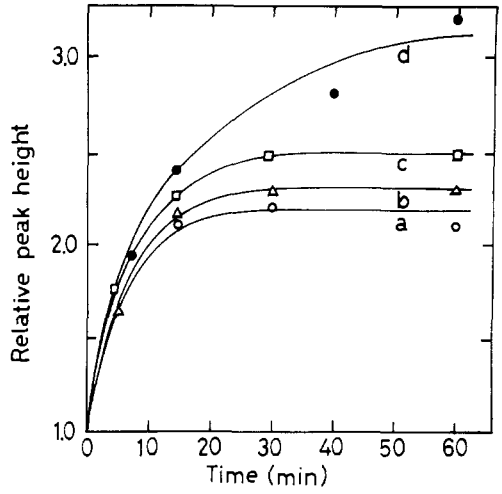

FIGURE 3: Time course of the spectral change of the mixture of HVJ* and RBC at 37°. HVJ was spin labeled by incubation at 30° for 1 hr of the mixture containing various concentrations of PC*: [PC*]/[viral phospholipid] = (a) 0.6, (b) 1.5, (c) 3, and (d) 6. The ordinate shows the central peak height on a relative scale.

same activities as those of intact virus. In most of the present experiments, HVJ* prepared by the incubation at 30° for 2 hr was used.

Transfer and Intermixing of PC between HVJ Envelope and RBC Membrane.* The ESR spectrum was unchanged when HVJ* was mixed with RBC at 0°. However, when the mixture was warmed to 37° where the virus-induced fusion occurred extensively, the spectrum dramatically changed (Figure 2). The peak height grew rapidly and the spectral shape changed. The peak height became from 2 to 3 times larger than the initial height after 30 min (Figure 3). HVJ* containing more concentrated PC* and exhibiting a more broadened spectrum showed a larger increase in the peak height. The peak height increase can be ascribed to disappearance of the exchange broadening due to dilution of PC*.

On further incubation at 37°, the spectrum gradually approached that of the host cell membrane spin labeled with PC* (compare Figure 4Aa with 4Ba). The conversion of the spectrum is not so clearly seen for the case of human RBC since the spectra of HVJ* and RBC* were rather similar to

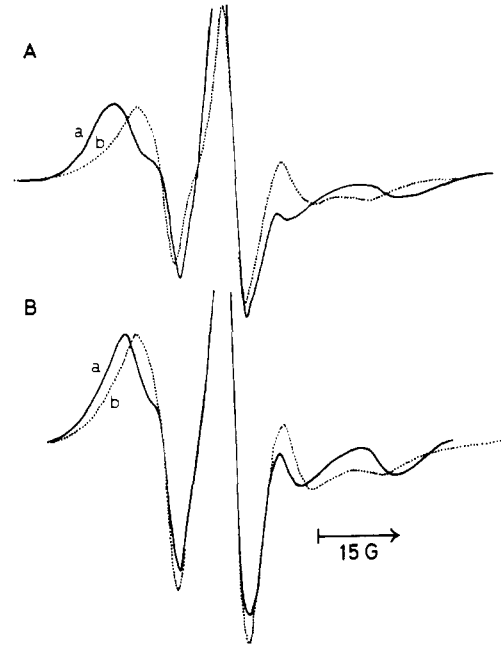

FIGURE 4: (A) ESR spectrum of spin-labeled human (a) and dace (b) RBC; (B) ESR spectrum of the mixture of HVJ* and human (a) and dace (b) RBC after incubation at 37° for 60 min. Spectra were measured at 20°.

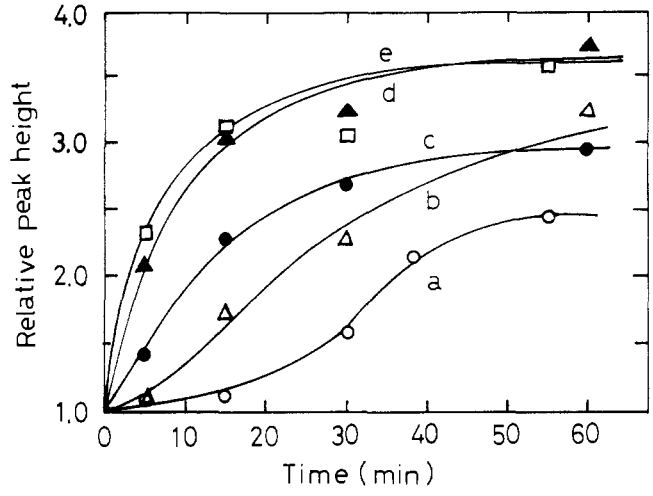

FIGURE 5: Temperature dependence of transfer of PC* from HVJ* to RBC. The mixture of HVJ* and RBC was incubated at (a) 19°, (b) 23.5°, (c) 28°, (d) 32.5°, and (e) 37°. The relative peak height was plotted against incubation time.

each other, both showing large overall splitting ($2T_{\parallel}$). The spectral conversion was more clearly demonstrated with RBC drawn from a fresh water fish. The dace RBC spin labeled with PC* showed a spectrum with markedly smaller overall splitting (44 G) than that of human RBC (54 G) (Figure 4A). The dace RBC therefore has a markedly fluid membrane compared with human RBC. When HVJ* was mixed with dace RBC and incubated at 37°, the spectral peak height increased rapidly in the initial stage and, after prolonged incubation, the spectrum tended to resemble that of dace RBC* (compare Figure 4Ab with 4Bb). HVJ was capable of inducing hemolysis of dace RBC.

The increase in the spectral peak height was also observed when nonlabeled HVJ was mixed with densely labeled RBC and incubated at 37°. The increase was 15–20%, much less than the case for HVJ* and RBC. All the

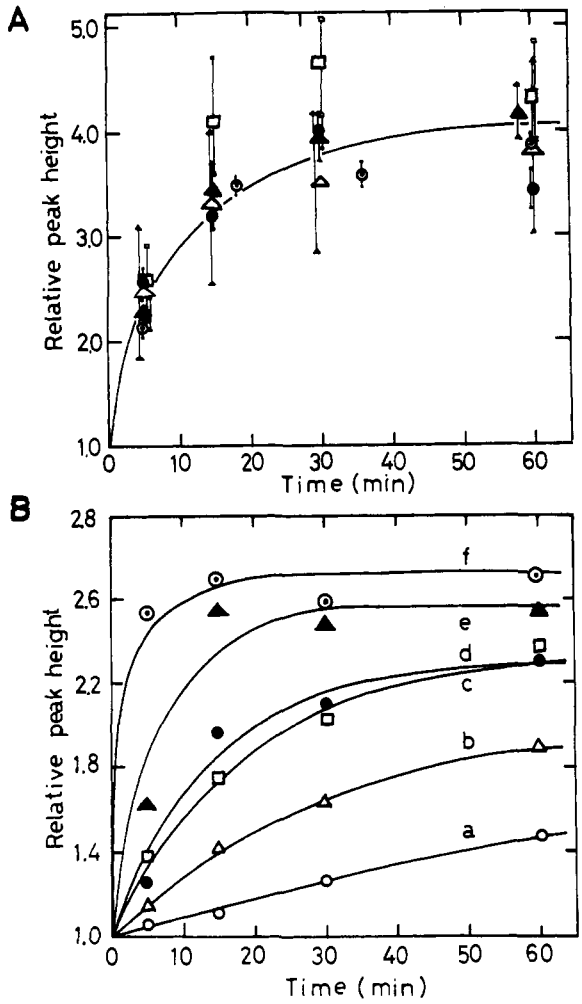

FIGURE 6: (A) Independence of the phospholipid transfer from HVJ to RBC of the viral dose. The reaction mixture contained 2.5% RBC and HVJ* at (△) 125, (□) 250, (●) 500, (▲) 1000, and (⊙) 1500 HAU/ml. Incubation at 37° (B) Dependence of HVJ-induced transfer of PC* between RBC on the viral dose. Densely labeled RBC* (0.8%) and nonlabeled RBC (1.7%) were incubated at 37° with HVJ at (a) 10, (b) 25, (c) 50, (d) 100, (e) 200, and (f) 500 HAU/ml.

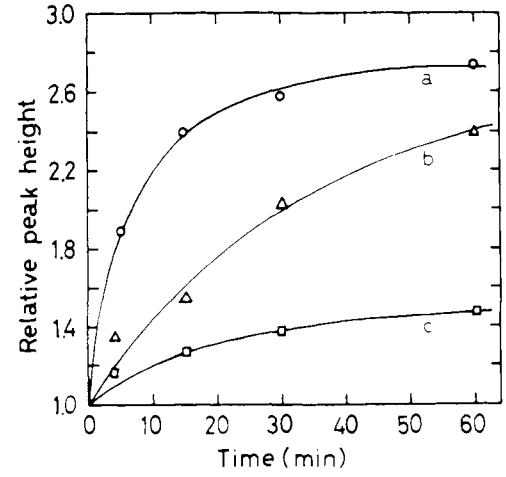

FIGURE 7: Effect of fusion inhibitors on the phospholipid transfer from HVJ* to RBC: (a) control; (b) in the presence of concanavalin A (25 μg/ml); (c) RBC was pretreated with 0.1% glutaraldehyde. The ESR peak height was plotted against incubation time at 37°.

above results clearly indicate that PC* molecules are rapidly transferred and intermixed between HVJ envelope and RBC membrane.

The transfer of PC* from the viral envelope to RBC did not occur at 0° where the virus adsorbed to RBC and caused aggregation but no fusion and hemolysis. The transfer reaction occurred vigorously at 37° where RBC was extensively fused. The incubation temperature thus has a profound effect on the transfer reaction. Results of a more detailed study on the temperature dependence are shown in Figure 5. The initial rate remained negligibly small until about 19° and rapidly increased between 25 and 30°. It should be noted that the S-shaped nature of the transfer reaction is evident at lower temperatures. The temperature dependence of the virus-induced fusion and hemolysis was similar to that of the transfer reaction.

The transfer rate of PC* was dependent on the fluidity of the host cell membrane, being larger for more fluid membranes. When HVJ* was incubated with crucian carp RBC, the initial increase in the peak height was far more rapid than that for human RBC. The fish RBC included a more fluid lipid bilayer as revealed by the ESR spectrum of the incorporated PC* ($2T_{\parallel}$ = 44 G at 23°).

The transfer reaction from HVJ* to RBC was independent of the viral dose (Figure 6A). The peak-height increase followed essentially the same time course within experimental error when various concentrations of HVJ* (125 to 1500 HAU/ml) were incubated with RBC (2.5%). Accordingly, the transfer proceeds in the direction of a zero-order reaction with respect to the virus concentration.

Effect of Fusion Inhibitors and Trypsin Treatment of HVJ. Since a close parallelism was observed between the phospholipid transfer and the virus-induced hemolysis and fusion, the effect of some fusion inhibitors on the transfer reaction was examined. Treatment of RBC with 0.1% glutaraldehyde at 37° for 20 min inhibited the virus-induced hemolysis and fusion completely. The ESR spectrum of a mixture of HVJ* and the modified RBC was measured at 37° as a function of incubation time. The increase in the peak height was greatly reduced, being only 19% of the control after 15 min (Figure 7c). Concanavalin A did not cause the virus-induced aggregates of RBC to fuse nor the aggregates to separate. It inhibited hemolysis considerably (K. Sekiguchi, unpublished). This reagent also retarded the increase of the spectral peak height (40% of the control after 15 min) (Figure 7b). The inhibition was to a lesser extent than that caused by glutaraldehyde treatment.

Trypsin digests the viral glycoprotein that has fusion and hemolysis activities but lacks hemagglutinating and neuraminidase activities. The enzyme does not modify the other glycoprotein, however, that possesses hemagglutinating and neuraminidase activities but lacks fusion and hemolysis activities (K. Shimizu, private communication). When the trypsinized HVJ* was incubated with RBC at 37°, the phospholipid transfer was greatly reduced. The peak-height increase was only 18% of the control after 15 min.

HVJ-Induced Transfer and Intermixing of PC between RBC.* When HVJ was added to a mixture of densely labeled RBC and nonlabeled RBC and the mixture was incubated at 37°, the exchange broadening of the ESR spectrum was rapidly and greatly reduced (Figure 8a). In the absence of HVJ, the spectral peak height increased only slightly. The results therefore indicate that the virus induced extensive transfer of PC* from RBC* to RBC. The transfer reaction was dependent on temperature in quite similar fashion to that for the virus-to-RBC transfer (Figure 8). No transfer

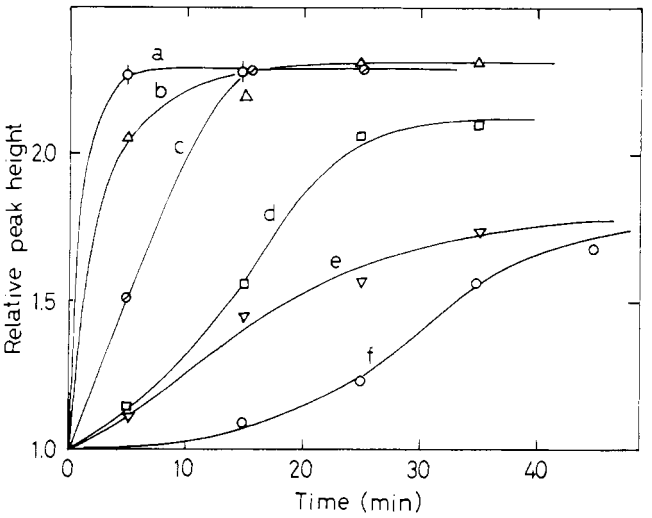

FIGURE 8: HVJ-induced transfer of PC* from RBC* to RBC and its temperature dependence. The mixture containing HVJ (1000 HAU/ml), RBC* (0.83%), and RBC (1.7%) was incubated at (a) 37°, (b) 28°, (c) 25°, (d) 22°, (e) 20.5°, and (f) 18.5°. The relative peak height was plotted against incubation time.

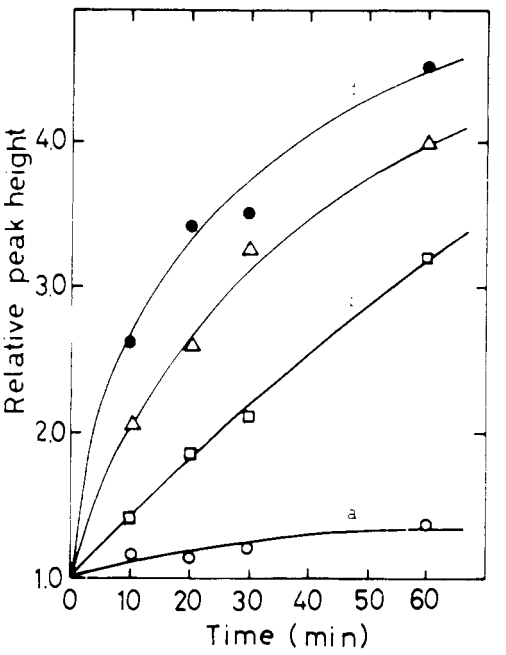

FIGURE 9: Transfer of PC* from spin-labeled influenza virus to RBC by the help of HVJ. Densely labeled influenza virus (1000 HAU/ml) and RBC (2.5%) were incubated at 37° in the presence of nonlabeled HVJ at (a) 0, (b) 200, (c) 800, and (d) 1500 HAU/ml. The ordinate plotted the ESR peak height measured at 20°.

was observed at 0°. The S-shaped nature of the transfer reaction was clearly observed at 18.5° (Figure 8f). Glutaraldehyde treatment of RBC completely abolished the virus-induced transfer of phospholipid between RBC. The transfer reaction was dependent on the viral dose (Figure 6B). The transfer rate was nearly proportional to the dose. The virus-induced fusion and hemolysis were also dependent on the dose. The hemolysis was found to be nearly proportional to the dose.

HVJ-Induced Transfer of PC from Influenza Virus to RBC.* The influenza virus A_0PR8 has hemagglutinating and neuraminidase activities but no fusion and hemolysis activities. It is therefore interesting to examine its capability of transferring PC* to RBC. When the densely labeled influenza virus was mixed with RBC and incubated at 37°, the spectral peak height showed only a slight increase, in great contrast to HVJ (Figure 9a). However, when nonlabeled HVJ was added to the mixture, the peak height increased greatly and rapidly. The increase was larger for the mixtures containing more concentrated HVJ (Figure 9b–d). These results clearly indicate that only a slight transfer of phospholipid occurred between the envelope of influenza virus and RBC membrane. Still more interesting is the finding that coexisting HVJ induced extensive transfer of PC* from influenza envelope to RBC membrane.

Discussion

The present study has demonstrated that the spin label is a useful tool for clarifying some of the molecular processes involved in the virus-induced cell fusion and provides a unique and quantitative measure for some key steps in the cellular reaction.

Phospholipid molecules can be transferred and intermixed between the viral envelope and the host RBC membrane in both directions. There exists some temperature-dependent process for the transmembrane movement. No transfer occurred at lower temperatures where the virus adsorbed to RBC and caused aggregation but no fusion and hemolysis. The transfer became appreciable at 19° and rapidly increased at higher temperatures. The rate-limiting process does not seem to be in the lateral diffusion of phospholipid molecules in the membranes.

Extensive transfer of phospholipid from virus to RBC was observed whenever the virus caused fusion and hemolysis, while the transfer was only slight or retarded under conditions of no or limited hemolysis and fusion. The close parallelism suggests that the virus-to-cell transfer is a necessary step to lead to fusion and hemolysis. The spin label thus provides an important parameter with regard to the earlier stage of the reaction. The viral proteins move to RBC membrane in the envelope fusion (Howe and Morgan, 1969; Bächi et al., 1973). The phospholipid transfer may be closely related to the protein movement, preceding and/or accompanying the movement. These molecular movements would modify the host cell membrane so as to be susceptible to fusion with virus and with cell. The S-shaped curve for the transfer reaction observed at lower temperature indicates that the initial molecular movements accelerate the following phospholipid transfer. The HVJ-induced extensive transfer of phospholipid from influenza virus to RBC also suggests such a modification. These are relevant to a morphological observation that the envelope fusion caused clustering of glycoproteins on RBC and brought about naked lipid surface (Bächi et al., 1973).

HVJ-induced transfer of phospholipid between RBC was also observed by the spin label. This phenomenon may be related to the cell fusion and can be used as another quantitative measure for a later stage of the reactions. This reaction followed the S-shaped curve at lower temperature in the same way as the virus-to-cell transfer, indicating that the same type of acceleration occurs in the intercellular transfer of phospholipid.

Acknowledgments

We are indebted to Dr. Tatsuo Iwasaki for providing influenza virus and to Professor Ryo Sato of Osaka University for his helpful discussions.

References

Bächi, T., Aguet, M., and Howe, C. (1973), *J. Virol. 11,* 1004.
Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 466.
Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biol. Physiol. 31,* 911.
Haywood, A. M. (1974), *J. Mol. Biol. 87,* 625.
Hosaka, H., and Shimizu, Y. K. (1972), *Virology 49,* 627.
Howe, C., and Morgan, C. (1969), *J. Virol. 3,* 70.
Hubbell, W. L., and McConnell, H. M. (1971), *J. Am. Chem. Soc. 93,* 314.
Lowry, O. H., Rosebrough, N. Y., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Maeda, T., and Ohnishi, S. (1974), *Biochem. Biophys. Res. Commun. 60,* 1509.
Okada, Y., and Murayama, F. (1968), *Exp. Cell Res. 53,* 34.
Poste, G., and Allison, A. C. (1973), *Biochim. Biophys. Acta 300,* 421.

The Self-Association of the Reduced ApoA-II Apoprotein from the Human High Density Lipoprotein Complex†

James C. Osborne, Jr.,* Giuseppe Palumbo, H. Bryan Brewer, Jr., and Harold Edelhoch

ABSTRACT: The molecular properties of the single linear chain form of human apoA-II, i.e., Cm apoA-II, have been evaluated by circular dichroism, polarization of fluorescence, difference absorption, and sedimentation equilibrium. The self-association of Cm apoA-II to a dimer resembles closely that of apoA-II though the free energy change is somewhat smaller. The dimerization of Cm apoA-II is accompanied by major changes in secondary and tertiary structure. The apoA-II molecule, therefore, represents a molecular association where the intramolecular structure is strongly dependent on the quaternary structure.

The two major protein components of the human high density lipoprotein complex (HDL)[1] are apoA-I and apoA-II, comprising 70 and 20%, respectively, of the proteins present. We have undertaken an investigation of the molecular properties of these proteins as a basis for understanding their behavior in HDL. We have reported that apoA-II self-associates to form a dimer (mol wt 34,760) at pH 7.4 with an association constant of $5 \times 10^4\ M^{-1}$ (Gwynne et al., 1975). The reaction was novel in that important changes in secondary and tertiary structure accompany the dimerization. Since apoA-II (mol wt 17,380) is a disulfide-linked dimer of a 77 residue chain (Brewer et al., 1972), it is possible to obtain the single chain by reduction of the disulfide bond. The liberated sulfhydryl group is subsequently protected by alkylation. Due to the structural changes involved in the self-association of apoA-II, it was of interest to compare the behavior of the single chain polypeptide with that of the double chain native molecule. The reduced molecule can also serve as a model for the apoA-II proteins of other species (rat and rhesus monkey) where apoA-II exists as a single chain which lacks cysteine residues (Herbert et al., 1974; Edelstein et al., 1973).

Methods

The preparation and purification procedures used for Cm apoA-II have been previously reported (Lux et al., 1972). It was shown by amino acid analysis that only the cysteine group is modified by reduction and alkylation with iodoacetic acid. Protein concentrations were determined by absorption at 280 nm employing a molar extinction coefficient of 5500 (calculated from amino acid analysis). A Radiometer pH meter was used for pH measurements. Glass redistilled water was used throughout and all chemicals employed were reagent grade with the exception of guanidine hydrochloride which was HEICO "Synthesized Extreme Purity".

Sedimentation equilibrium experiments were performed in a Spinco Model E ultracentrifuge equipped with a temperature control system and a photoelectric ultraviolet scanner. The double sector cells, containing epon filled aluminum centerpieces and quartz windows, were centrifuged in an ANG-Ti rotor. The layering fluid was omitted. Molecular weights were obtained by using the following equation:

$$M_W^{app} = \frac{2RT}{\omega^2(1 - \bar{\nu}\rho)} \frac{d \ln c}{dr^2} \quad (1)$$

where M_W^{app} is the apparent weight average molecular weight; R, the gas constant; T, the absolute temperature; ω, the angular velocity; $\bar{\nu}$, the partial specific volume; ρ, the solvent density; c, the protein concentration; and r, the distance from the center of rotation. For a monomer-dimer equilibrium eq 1 reduces to

$$C_r = C_{1,m}e^{AM_1(r^2-m^2)} + C_{2,m}e^{2AM_1(r^2-m^2)} \quad (2)$$

where C_r is the total concentration at distance r from the center of rotation; $C_{1,m}$ and $C_{2,m}$ are the concentrations of monomer and dimer at the meniscus; $(r^2 - m^2)$ corresponds to the radial distance from the meniscus (m); M_1 is the monomer molecular weight and $A = \omega^2(1 - \bar{\nu}\rho)/2RT$.

† From the Clinical Endocrinology Branch, NIAMDD, and the Molecular Disease Branch, NHLI (H.B.B., Jr.), National Institutes of Health, Bethesda, Maryland 20014. *Received April 3, 1975.* This work was supported by a grant to G.P. from the Kroc Foundation, Santa Ynez, California.

[1] Abbreviations used are: HDL, high density lipoprotein; Cm apoA-II, reduced and carboxymethylated apoA-II; Gdn-Cl, guanidine hydrochloride.