Campbell, I. M. (1979) *Anal. Chem. 51*, 1012A–1019A.
Campbell, I. M., Coscia, C. J., Kelsey, M., & Bentley, R. (1967) *Biochem. Biophys. Res. Commun. 28*, 25–29.
Campbell, I. M., Robins, D. J., Kelsey, M., & Bentley, R. (1971) *Biochemistry 10*, 3069–3078.
Cox, G. B., & Gibson, F. (1964) *Biochim. Biophys. Acta 93*, 204–206.
Cox, G. B., & Gibson, F. (1966) *Biochem. J. 100*, 1–6.
Dansette, P., & Azerad, R. (1970) *Biochem. Biophys. Res. Commun. 40*, 1090–1095.
Grotzinger, E., & Campbell, I. M. (1974) *Phytochemistry 13*, 923–926.
Guérin, M., Leduc, M. M., & Azerad, R. G. (1970) *Eur. J. Biochem. 15*, 421–427.
Hutson, K. G., & Threlfall, D. R. (1978) *Biochim. Biophys. Acta 530*, 1–8.
McGovern, E. P., & Bentley, R. (1978) *Arch. Biochem. Biophys. 188*, 56–63.
Meganathan, R., & Bentley, R. (1979) *J. Bacteriol. 140*, 92–98.
Meganathan, R., Folger, T., & Bentley, R. (1980) *Biochemistry 19*, 785–789.
Meganathan, R., Bentley, R., & Taber, H. (1981) *J. Bacteriol. 145*, 328–332.
Robins, D. J., & Bentley, R. (1972) *J. Chem. Soc., Chem. Commun.*, 232–233.
Robins, D. J., Campbell, I. M., & Bentley, R. (1970) *Biochem. Biophys. Res. Commun. 39*, 1081–1086.
Robins, D. J., Yee, R. B., & Bentley, R. (1973) *J. Bacteriol. 116*, 965–971.
Samuel, O., & Azerad, R. (1972) *Biochimie 54*, 305–317.
Shineberg, B., & Young, I. G. (1976) *Biochemistry 15*, 2754–2758.
Stroobant, P., Young, I. G., & Gibson, F. (1972) *J. Bacteriol. 109*, 134–139.
Young, I. G. (1975) *Biochemistry 15*, 399–406.

Interaction of Hemagglutinating Virus of Japan with Erythrocytes As Studied by Release of a Spin Probe from Virus†

Toyozo Maeda,‡ Kazumichi Kuroda, Sakuji Toyama, and Shun-ichi Ohnishi*

ABSTRACT: The spin probe tempocholine was incorporated into hemagglutinating virus of Japan (HVJ) and its release on interaction with erythrocytes or ghosts studied. Two methods were used for the assay of release. One is to incorporate the probe into virus at a high concentration (~30 mM) and use the increase in the ESR peak height due to drastic weakening of the spin–spin exchange interaction on dilution. The other is to load the virus with a low concentration of tempocholine. The tempocholine remaining inside the virus was determined by reducing outside tempocholine with ascorbate. The results showed that tempocholine was rapidly released on interaction with both erythrocytes and ghosts. Requirement of the viral F glycoprotein for the release was demonstrated by using trypsinized HVJ, which has cleaved F protein, and also by using HVJ grown in cultured cells, which has the precursor form of F protein. The release was independent of the viral dose and occurred to the same extent with intact erythrocytes as with ghosts. The release from trypsinized HVJ was not enhanced by coadsorbed intact HVJ. These characteristics for the release were markedly different from those for the phospholipid transfer from HVJ envelope to erythrocyte membrane. Combining the results for the spin probe release and the phospholipid transfer has suggested that the envelope fusion is not autocatalytically accelerated, is independent of the number of virus adsorbed on the cell, and occurs with ghosts as efficiently as with intact erythrocytes. HVJ causes some modification of the target cell membrane and enhances phospholipid transfer from the envelope to erythrocyte membrane through the modification. In ghosts, the modification is localized and no enhancement occurs. A model was put forward for the enhancement based on cell swelling; envelope fusion causes an increase in the permeability of target cell membrane and results in cell swelling. The swelling causes modification of the cell membrane which is responsible for the enhancement.

Membrane fusion is associated with many important cellular functions such as endocytosis, secretion of synthesized products from cells, and bulk transport [see Poste & Nicolson (1978)]. HVJ,[1] synonym of Sendai virus, has been used as a simple and reproducible system for the study of fusion mechanisms under controlled conditions, and much information has been accumulated [see Ishida & Homma (1978)]. The enveloped virus has two kinds of membrane glycoproteins, called HANA and F. HANA is responsible for hemagglutinating and neuraminidase activities, while F is essential for hemolytic and fusion activities. The virus first binds to the cell surface, causing gigantic aggregates of cells. The viral envelope then fuses to the target cell membrane, followed by hemolysis and finally cell fusion [see Poste & Nicolson (1977)]. Interrelationships between envelope fusion, hemolysis, and cell fusion are not yet fully understood. Biophysical methods should be able to give detailed molecular insights into the fusion process.

† From the Department of Biophysics, Faculty of Science (T.M., K.K., and S.O.), and Department of Serology and Immunology, Institute for Virus Research (S.T.), Kyoto University, Kyoto, Japan 606. *Received August 14, 1980.* This work was supported by Grant-in-Aid No. 411304 for Special Project Research, Biomembranes, The Ministry of Education, Science and Culture, Japan.
‡ Deceased Jan 8, 1981.

[1] Abbreviations used: HVJ, hemagglutinating virus of Japan; HAU, hemagglutinating unit; tempocholine, *N,N*-dimethyl-*N*-(2,2,6,6-tetramethyl-4-piperidinyl)-*N*-(2-hydroxyethyl)ammonium chloride; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/81/0420-5340$01.25/0

We have been studying the virus–cell interaction using a spin-labeled phospholipid and found a rapid F protein-dependent transfer of phospholipid from viral envelope to erythrocyte membrane and between erythrocyte membranes (Maeda et al., 1975, 1977). The phospholipid transfer becomes greater with an increase in the amount of virus particles adsorbed on the cell. The transfer from trypsinized HVJ to erythrocyte membrane was very low because of the absence of the active F protein. However, when intact HVJ was added at the same time, the transfer from trypsinized virus was greatly enhanced and became larger with an increase in the amount of intact HVJ. Ghosts are markedly different from erythrocytes in the interaction with HVJ. The phospholipid transfer to ghost membranes was smaller and only slightly dependent on the viral dose. Intact HVJ did not enhance the transfer from trypsinized HVJ to ghost membranes. The results indicated a cooperative nature in the interaction between HVJ and intact erythrocytes mediated by the target cell membrane but not between HVJ and ghosts (Kuroda et al., 1980).

The previous studies raised several interesting questions on the virus–cell interaction. The present study is mainly directed to one of them. Is the enhancement of phospholipid transfer a result of enhancement of envelope fusion or due to enhancement of the transfer itself without causing envelope fusion? It is clear that if envelope fusion were enhanced, the transfer should also be enhanced since the phospholipid molecules can rapidly diffuse from the fused site in the target cell membrane. To examine this question, we studied the virus–cell interaction by measuring the release of a small molecule tempocholine previously incorporated into the HVJ. The release is closely related to envelope fusion since the viral contents are released into cells on envelope fusion. Virus particles were loaded with the spin probe to a high concentration, and the release was followed by measuring the peak height increase of the ESR spectrum. This provides a rapid, sensitive, in situ assay method for the release based on the concentration dependence of ESR spectrum [see Maeda et al. (1978)]. As an alternative assay method, the spin probe remaining inside the virus particles was measured by reducing the outside tempocholine nitroxide moiety by ascorbate. The two methods gave data consistent with each other. The results of the present study show that tempocholine release represents envelope fusion, requiring viral F glycoprotein, and has a characteristic difference from the phospholipid transfer reaction. Combining the results of phospholipid transfer and spin probe release has led to several interesting conclusions on the initial stage of virus–cell interaction.

Materials and Methods

Virus. HVJ, z strain, was used throughout unless otherwise indicated. The virus was grown for 72 h in the allantonic cavity of 10-day-old eggs and purified as described previously (Maeda et al., 1975). Inactivation of HVJ was carried out by treatment with 30 μg/mL trypsin (grade III, Miles-Seravac) for 20 min at 37 °C; the action of trypsin was stopped by addition of soybean trypsin inhibitor (120 μg/mL, Miles-Seravac). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that this treatment selectively digested only F protein, greatly decreasing hemolysis and fusion activities without almost no loss of hemagglutinating and neuraminidase activities, in agreement with Shimizu & Ishida (1975). HVJ, Fushimi strain, was also used in some experiments by growing the virus in a monolayer culture of $LLCMK_2$ cell line. Activation of this virus was carried out by treatment with 5 μg/mL trypsin for 7.5 min at 37 °C, followed by the addition of soybean trypsin inhibitor (20 μg/mL) according to Homma (1972). Viruses were stored at −70 °C. The assay of hemagglutinating activity was done by the pattern method (Salk, 1944). Virus concentration was expressed as HAU/mL.

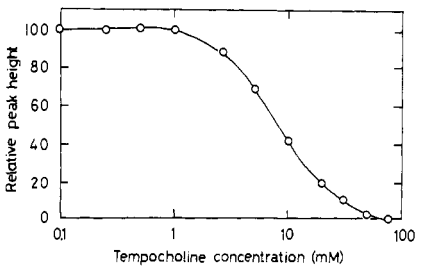

FIGURE 1: Concentration dependence of ESR peak height of tempocholine in water. The low-field peak height of the spectrum at 37 °C normalized to unit double-integrated area is plotted.

Erythrocytes and Ghosts. Fresh human blood was obtained from a local blood bank and used within 2 weeks. Blood was rendered buffy coat free by washing 3 times with phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$) and resuspended in the same buffer (10% v/v). Washed erythrocytes were lysed in 40 volumes of cold 5 mM sodium phosphate buffer (pH 8), washed once, resuspended in 10 volumes of warm phosphate-buffered saline, and incubated for 1 h at 37 °C for resealing (Steck, 1974). The ghosts were then spun down and resuspended in the same buffer. Treatment of erythrocytes with glutaraldehyde (0.1%) was performed at 37 °C for 20 min.

Loading Virus Particles with Tempocholine. HVJ (2.5×10^4 HAU) was suspended in 0.5 mL of 140 mM or 1 mM tempocholine buffered at pH 7.6 with Tris-HCl and incubated at 30 °C for 4 h. The virus was then washed 3 times with phosphate-buffered saline at 4 °C and resuspended in 1 mL of buffer. The higher concentration of tempocholine was used to load large amounts of tempocholine for the assay of release, while the lower concentration of tempocholine was used for loading small amounts of tempocholine to measure tempocholine remaining in the virus particles. The amount of incorporation was determined from the ESR signal intensity after the virus was treated with 1% Triton.

Assay of Tempocholine Release from Preloaded Virus Particles. Tempocholine-loaded HVJ (20 μL, 300–12000 HAU/mL) was mixed with 1 mL of erythrocytes or ghosts (3% v/v) at 0 °C and, after 10 min, centrifuged for 3 min at 350*g*. The resulting pellet was placed in a quartz capillary tube and inserted into the ESR spectrometer cavity, and the ESR spectrum was recorded repeatedly at 37 °C. Release of tempocholine was measured by the increase in the peak height of ESR spectrum. Since the virus was loaded with a high concentration of tempocholine (~30 mM; see Results), the ESR spectrum of virus is broadened due to strong spin–spin exchange interaction. Therefore, when tempocholine leaks out of the virus and becomes diluted, the exchange interaction is weakened, and the broadening becomes greatly diminished. This causes an increase in the ESR peak height. A model experiment with tempocholine aqueous solution to determine concentration dependence of the peak height is shown in Figure 1. From Figure 1, the peak height increases by a factor of 10 when 30 mM tempocholine is diluted to a concentration lower than 0.2 mM.

As an alternative assay for the release, the amount of tempocholine remaining in the virus particles was measured. This was done by adding 1 mM ascorbate to the virus–cell suspension since it immediately (in less than 1 min) reduces the

Table I: Incorporation of Tempocholine into HVJ and Its Release from the Loaded Virus on Incubation with Erythrocytes

incubation for loading of tempocholine[a]		amount of incorporation[b] (pmol/HAU)	release of tempocholine from virus[c]
temp (°C)	time (h)		
30	1	3.3	1.4
	2	5.0	2.0
	4	7.1	4.4
	6	9.4	3.9
	14	ND[d]	2.8
4	14	2.4	1.9
20	14	4.2	3.5
37	6	8.4	3.5

[a] HVJ (5×10^4 HAU/mL) was incubated with 140 mM tempocholine. [b] Determined by comparing the ESR signal intensity of the virus suspension solubilized by 1% Triton with that of a known concentration of tempocholine in the Triton solution. [c] Increase in ESR peak height after incubation with ghosts. The loaded virus was mixed with resealed ghosts at 4 °C, and the ESR spectrum of the virus–cell aggregates was measured at 37 °C. The central peak height after 15 min divided by that at time 0 is given. [d] ND, not determined.

nitroxide moiety of accessible tempocholine and causes disappearance of the ESR signal. On the other hand, tempocholine inside the virus particle is protected from the externally added ascorbate. Reduction of the inside tempocholine took a much longer time ($T_{1/2}$ = 53 min at 37 °C). The lightly loaded HVJ was used for this assay since it gives practically no spin–spin broadening in the spectrum and therefore the peak height is linearly dependent on the concentration of tempocholine in the virus.

Results

Incorporation of Tempocholine into HVJ. HVJ readily incorporated tempocholine. The amount of incorporation increased with incubation time (Table I). For example, when HVJ (5×10^4 HAU/mL) was incubated with tempocholine (140 mM) at 30 °C, the amount of incorporation per HAU was 3.3 pmol after 1 h and 5.0 pmol after 2 h. The ESR spectrum of loaded virus became broadened on longer incubation due to increased spin–spin exchange interaction. The broadening is already noticeable after 1-h incubation and becomes very large after 4-h incubation. The spectrum after 4-h incubation was similar to a model spectrum for aqueous tempocholine at 20–30 mM. The amount of incorporation decreased at lower temperatures (Table I). Therefore, incubation at 4 °C, which appears preferrable for keeping the virus intact, did not give the amount of incorporation suitable for the assay of release. In the following experiments, incubation at 30 °C for 4 h was chosen as optimum.

The ready and extensive incorporation of tempocholine is probably due to its positive charge through which it may interact with negatively charged groups such as nucleic acids inside the virus. In fact, incorporation of a neutral spin probe, 4-hydroxy-2,2,6,6-tetramethyl piperidinyl-1-oxy, and a negatively charged spin probe, 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy, was much lower than that of tempocholine (0.01 pmol and 0.2 pmol per HAU after 4 h at 30 °C, respectively). We estimated the rotational correlation time of tempocholine in the virus from the ESR peak height ratios; the result was 5.7×10^{-11} and 1.1×10^{-10} s, depending on which peaks were used for the calculation (Stone et al., 1965). The correlation time was larger than 1.3×10^{-11} s for aqueous tempocholine at 37 °C, indicating some restriction of the rotational motion in the virus. The different values depending on the peaks suggest its anisotropic nature. On the other hand, the rotational correlation time for the neutral and negative spin probes was $\sim 10^{-11}$ s irrespective of whether they were in water or in the virus.

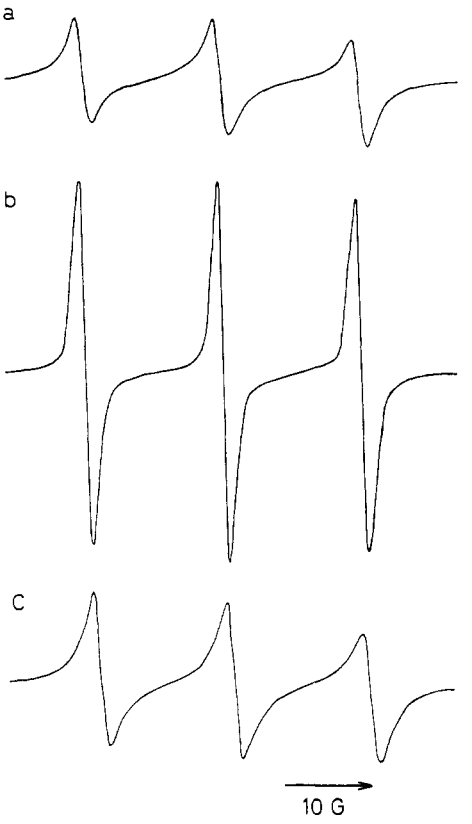

FIGURE 2: Change in the ESR spectrum of tempocholine-loaded HVJ on incubation with erythrocyte ghosts at 37 °C: (a) 0 min and (b) 15 min. Spectrum c is obtained after ascorbate addition (1 mM) to the sample (b). Relative gain of the ESR spectrometer is 1:1:3.3 for (a–c). Loading of virus was carried out by incubation with 140 mM tempocholine for 4 h at 30 °C.

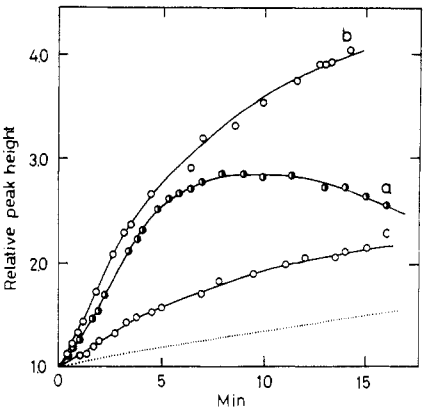

FIGURE 3: Tempocholine release from virus on incubation with erythrocytes or ghosts at 37 °C: (a) HVJ–erythrocytes, (b) HVJ–ghosts, and (c) trypsinized HVJ–ghosts. Loading of virus was carried out by incubation with 140 mM tempocholine for 4 h at 30 °C. ESR spectrum of the virus–cell aggregates was measured continuously at 37 °C, and the ratio of the central peak height at time t to that at time 0 (I_t/I_0) is plotted. The dotted line shows spontaneous leakage from loaded virus in the absence of cells.

Rapid Release of Tempocholine from HVJ. When the tempocholine-loaded HVJ was added at 4 °C to ghosts and the resulting virus–cell aggregates were incubated at 37 °C, the ESR spectrum changed rapidly from an exchange-broadened signal to a sharp one (Figure 2). The peak height increased by a factor of 4 after 15 min (Figure 3b). This

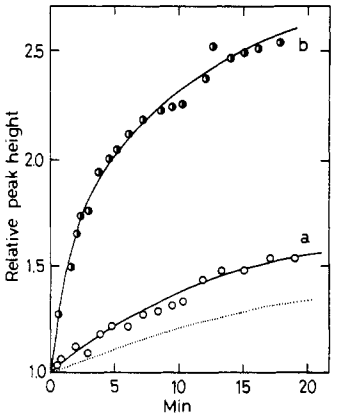

FIGURE 4: Tempocholine release from virus on incubation with ghosts at 37 °C. HVJ grown in the cultured cell was used intact (a) and after activation by light trypsinization (b). Increase in the hemolysis activity was from 14% to 70% hemolysis after incubation with 700 HAU/mL virus for 60 min at 37 °C. The dotted line represents spontaneous leakage from the virus. The ordinate plots the ESR peak height increase.

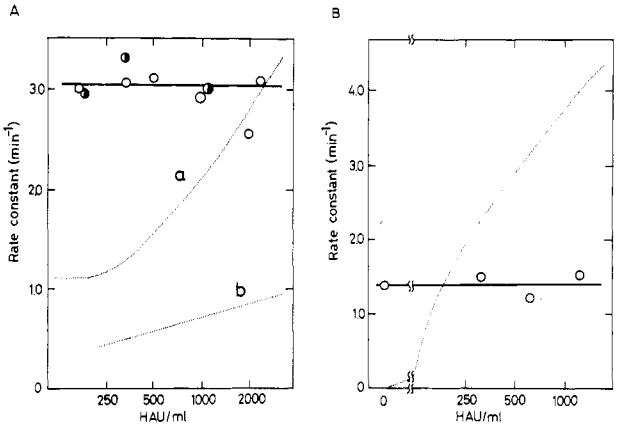

FIGURE 5: (A) Rate constant of tempocholine release from HVJ on incubation with erythrocytes (O) or resealed ghosts (◑) as a function of virus concentration. The rate constant was obtained from the initial slope of the curve for ESR peak height increase vs. incubation time. The value for erythrocytes was corrected for the nitroxide reduction by hemolyzate as described in the text. The dotted lines show the amount of phospholipid transferred in 10 min at 37 °C to erythrocytes (a) or ghosts (b), taken from Kuroda et al. (1980). When the reactions were studied in earlier stage (~2 min), the difference between erythrocytes and ghosts was smaller. (B) Rate constant of tempocholine release from trypsinized HVJ on incubation with ghosts at 37 °C. Tempocholine-loaded trypsinized HVJ (400 HAU/mL) and various concentrations of unloaded intact HVJ were adsorbed on ghosts and incubated at 37 °C. The abscissa plots concentration of the coadsorbed intact HVJ. The dotted line shows the amount of phospholipid transfer from trypsinized HVJ to erythrocytes in 10 min at 37 °C as a function of coadsorbed intact HVJ (Kuroda et al., 1980).

change indicates a rapid release of tempocholine from the preloaded virus. When ascorbic acid was added to the sample after 15 min, most of the sharp signal disappeared, and a broad signal similar to the original one remained with about half of the initial strength (Figure 2c). Spectrum b is therefore a superposition of the sharp and the broad signal. The signal reduction in Figure 2c indicates that about 50% of tempocholine was released during the 15-min incubation at 37 °C. A more accurate measurement of the trapped tempocholine, using the lightly loaded virus, also showed 45% of the tempocholine remaining trapped inside the virus after 15-min incubation at 37 °C (see a later section). The results could reasonably suggest that there is complete release of tempocholine from 55% of the adsorbed virus particles and almost no release from the rest of particles.

The effect of different conditions for tempocholine incorporation on the peak height increase is shown in Table I. It is reasonable to observe larger increases for the virus incubated with tempocholine for longer periods. However, when the incubation was too long, the peak height increase became even smaller in spite of the larger concentration of incorporated tempocholine; compare the 4.4 times increase for 4-h incubation at 37 °C with the 3.9 times increase for 6-h incubation. This may be due to gradual inactivation of HVJ during incubation at this temperature.

Release of Tempocholine Requires Active F Protein. Since F protein is included in phospholipid transfer as well as envelope fusion, hemolysis, and cell fusion, we investigated whether it is necessary in tempocholine release. In one experiment, trypsinized HVJ was used. The trypsinized virus incorporated as much tempocholine as intact virus. Spontaneous leakage from the virus was also the same as that from intact virus. When the tempocholine-loaded trypsinized virus was added to ghosts and incubated at 37 °C, the ESR peak height increase was small, only a little larger than spontaneous leakage (Figure 3c). This shows that active F protein is an essential requirement for the rapid release of tempocholine.

In other experiments, HVJ grown in cultured cells, which contains normal HANA but a precursor form of F protein (F_0), was used. A brief treatment with trypsin splits F_0 into two fragments and activates hemolysis and fusion activities (Homma & Ohuchi, 1973). The tempocholine release from HVJ grown in cultured cells was small (Figure 4a), while the release from the activated virus was much greater (Figure 4b). The results again indicate that active F protein, but not the F_0 form, is required for the release. Binding and aggregation of erythrocytes by HANA proteins by themselves are not enough to cause the release.

Same Rate of Release Using Erythrocytes or Ghosts. The assay of tempocholine release in the preceding sections was done with ghosts as target cells. When intact erythrocytes were used as the target, the ESR peak height increase was as rapid as that for ghosts in the initial phase, but after some time interval (6–8 min), the peak height stopped increasing and thereafter decreased (Figure 3a). The decrease of peak height in the later stage is due to reduction of the tempocholine nitroxide group by some reducing agent(s) in the erythrocyte cytoplasm. In fact, freshly prepared hemolyzate rapidly reduced tempocholine, causing decay of the ESR signal to about half in 6.5 min at 37 °C. The reduction of tempocholine followed the first-order kinetics with a rate constant of 0.25 min^{-1} at 37 °C in a concentration range up to 0.2 mM tempocholine.

The tempocholine release from HVJ by interaction with erythrocytes was quite similar to that of interaction with ghosts except for the nitroxide reduction. For quantitative comparison, we estimated the rate of release from the initial slope with a correction factor based on the assumption that all tempocholine released is dilute enough to give the peak height without exchange broadening and reduced by hemolyzate at a rate constant of 0.25 min^{-1}. The tempochloline remaining gives the initial exchange-broadened spectrum. The rate of tempocholine release calculated on this assumption agrees well with that for ghosts (see Figure 5A). As shown in a later section, the assay of trapped tempocholine in virus particles after incubation gives clearer data on this point.

The same rate of release on interaction with erythrocytes and ghosts is markedly different from the degree of phospholipid transfer to erythrocytes as compared to ghosts.

Independence of Tempocholine Release of Viral Dose. Tempocholine release from HVJ on incubation with erythro-

cytes or ghosts was investigated by changing the virus concentration over a wide range from 80 to 1900 HAU/mL. The curves for the ESR peak height increase vs. incubation time were almost superimposable and quite similar to Figure 3b for ghosts and Figure 3a for erythrocytes. The rate constant of release was obtained from the intial slope of the curves and plotted in Figure 5A. The data clearly show that the release reaction is independent of the viral dose and also the similarity between erythrocytes and ghosts in the reaction. These characteristics for the release are in marked contrast to the phospholipid transfer which was greatly dependent on the dose for erythrocytes and only slightly dependent in the case of ghosts, as reproduced by dotted lines in Figure 5A.

HVJ Does Not Enhance Tempocholine Release from Trypsinized HVJ. Tempocholine release from trypsinized HVJ was very low, as described in a preceding section. Whether active HVJ can enhance the release was investigated by adsorbing both tempocholine-loaded trypsinized HVJ and unloaded intact HVJ on erythrocyte membranes. The results show no enhancement of release from trypsinized HVJ by active HVJ. The rate constant of release was independent of the amount of coadsorbed intact HVJ (Figure 5B). This result again shows the absence of membrane-mediated enhancement in the release reaction, in marked contrast to the phospholipid transfer which was greatly accelerated by intact HVJ (see dotted line in Figure 5B).

Assay of Unreleased Tempocholine by Ascorbate Reduction. As a complementary assay of release, tempocholine remaining in the virus particles was measured by reducing tempocholine outside the particles with ascorbate. Ascorbate can reduce the accessible nitroxide moiety almost immediately but cannot penetrate the viral envelope within the time period of the assay. The lightly loaded virus was used for this assay. The decrease in the ESR peak height was followed when the virus–cell aggregates were incubated in the presence of ascorbate.

Figure 6 shows some results of such an assay; the fraction of unreleased tempocholine is plotted against time of incubation with erythrocytes (A) or ghosts (B). It is shown that tempocholine released rapidly from virus, initially following an exponential curve. The amount of release reached 50–55% after 15 min both for erythrocytes and for ghosts. The release proceeded in the same way with erythrocytes and ghosts and was independent of viral dose. Figure 6C shows release from trypsinized HVJ over incubation time. The release was slow (~15% after 15 min) and not accelerated by active HVJ coadsorbed. These results are in complete agreement with those obtained by the peak height increase assay due to dilution of tempocholine. The assay of unreleased tempocholine may be particularly useful for the study of interaction with cells whose cytoplasm has reducing activity.

Glutaraldehyde Treatment of Erythrocytes Inhibits Release from HVJ. HVJ caused agglutination of glutaraldehyde-treated erythrocytes or ghosts, and viral neuraminidase functioned to hydrolyze the receptor sialic acid. However, the virus did not cause hemolysis and fusion of glutaraldehyde-treated erythrocytes. When tempocholine-loaded HVJ was incubated with glutaraldehyde-treated ghosts at 37 °C, the ESR peak height increased only to a small extent (data not shown). The release was therefore limited. These results suggest that some mobility of target cell membrane components is necessary for the functioning of F protein to cause the rapid release of tempocholine as well as hemolysis and fusion.

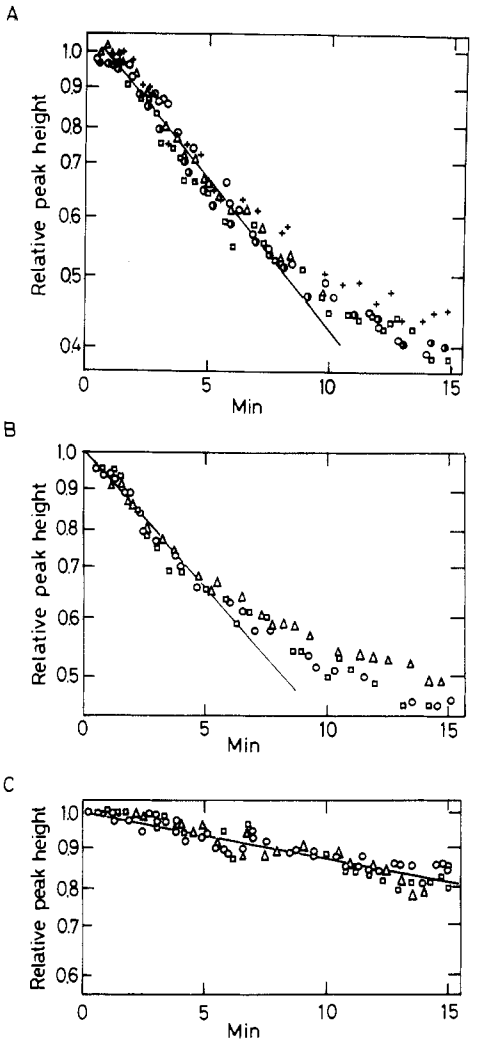

FIGURE 6: Assay of tempocholine remaining trapped inside HVJ on incubation with erythrocytes (A) or resealed ghosts (B). Loading of tempocholine was carried out by incubation with 1 mM tempocholine for 4 h at 30 °C. The lightly loaded HVJ was incubated at 37 °C with erythrocytes or ghosts in the presence of 1 mM ascorbate, and the ESR spectrum was measured continuously. The peak height is proportional to the concentration of tempocholine. Various symbols represent data points obtained with different concentrations of HVJ: 104 (+), 208 (□), 416 (○), 832 (△), and 1248 HAU/mL (●). (C) Tempocholine remaining trapped inside trypsinized HVJ on incubation with erythrocytes. The lightly loaded trypsinized HVJ (390 HAU/mL) and various concentrations of intact HVJ were added to erythrocytes and incubated at 37 °C: 0 (○), 312 (□), and 1248 HAU/mL (△).

Discussion

The present study clearly shows that the release of a small molecule from virus gives valuable information about the early stage of virus–cell interactions. The phospholipid transfer between virus and cell membranes has also given important unique information on the interaction (Maeda et al., 1975, 1977; Kuroda et al., 1980). Both reactions required viral F glycoprotein but had a characteristic difference. There was a marked acceleration of phospholipid transfer mediated by the target cell membranes but no such enhancement in the release.

It is obvious that the envelope fusion of tempocholine-loaded virus with cell membranes accompanies release of tempocholine. Another possibility also exists for the release, increased permeability of the viral envelope on binding to and interacting with cell membranes. Deformation of envelope and rearrangement of envelope proteins have been observed (Knutton, 1976, 1977). Such events could change the permeability of the envelope. At present, we do not know what fraction of the release is due to the permeability change, if any.

However, it is important to note that if the envelope fusion occurs, the tempocholine release necessarily follows. It is therefore reasonable to assume that if the degree of the release is unchanged, then the degree of envelope fusion is on the same level.

The release of tempocholine from trypsinized HVJ is small, and the coadsorbed intact HVJ does not enhance the release. This result indicates that intact HVJ does not cause envelope fusion of trypsinized virus with cell membrane. On the other hand, the phospholipid transfer from trypsinized HVJ to erythrocytes was small but greatly enhanced by the coadsorbed intact HVJ. It is therefore concluded that the intact HVJ enhances only the phospholipid transfer from trypsinized virus without causing enhancement of envelope fusion. The modification of the target cell membrane induced by active HVJ causes enhancement of phospholipid transfer or exchange between trypsinized envelope and cell membrane.

The F-protein-dependent release of tempocholine was much larger than the release from trypsinized HVJ. This release is independent of the viral dose, again in marked contrast to the phospholipid transfer to erythrocytes which is greatly enhanced by increasing amounts of adsorbed HVJ. The dose-dependent enhancement is therefore enhancement of phospholipid transfer itself but not due to enhancement of envelope fusion. In other words, the envelope fusion is not autocatalytically accelerated through modification of the target cell membrane. The probability of envelope fusion should therefore be a constant, independent of the number of adsorbed virus particles. In our previous paper (Kuroda et al., 1980), enhancements of envelope fusion and phospholipid transfer were taken into consideration as the possible cause for the enhanced transfer, but we can now disregard the former.

The tempocholine release was almost the same for erythrocytes and ghosts. This result suggests that envelope fusion occurs to the same extent with erythrocytes and ghosts, in agreement with the results of Lyles & Landsberger (1979). On the other hand, the phospholipid transfer was quite different between erythrocytes and ghosts. The transfer to ghosts was small and only slightly dependent on the viral dose, while the transfer to erythrocytes was large and greatly enhanced when viral dose was increased. What makes ghosts different from erythrocytes is the lack of a large amplification of the virus-induced modification of target cell membrane so that no enhancement of phospholipid transfer may occur. Using spin-labeled phosphatidylcholine but on the basis of a different assay principle, Lyles & Landsberger (1979) showed no enhancement of phospholipid transfer to erythrocytes and the same rate of transfer to erythrocytes as to ghosts, inconsistent with our previous data (Kuroda et al., 1980). We think that the enhancement does occur and is not an artifact due to increased membrane fluidity of ghosts which causes an increase in the ESR peak height. If we take into account the increase in the peak height in ghost membranes, then the same rate of transfer to erythrocytes and ghosts would mean slower transfer to ghosts.

What is the modification and its propagation or amplification in erythrocyte membrane? We have previously proposed that lateral diffusion of viral F proteins in the target cell membrane is responsible for the modification and propagation. The viral proteins in the cell membranes could cause enhancement of phospholipid transfer or exchange between the virus and cell membranes. It was shown that lateral diffusion of viral proteins in the fused erythrocyte membranes actually occurs with the diffusion constant of the same order of magnitude as the propagation rate of modification, 4×10^{-11} $cm^2\ s^{-1}$ (Kuroda et al., 1980).

Knutton (1977, 1979) and Knutton & Bächi (1980) have recently emphasized the importance of osmotic swelling in cell fusion; the swelling is a driving force by which the membrane sites of envelope fusion expand to form polyerythrocytes. The early harvested 1-day-old HVJ, which was shown to be nonhemolytic (Homma et al., 1976), does not induce cell swelling and, hence, polyerythrocyte formation. We now put forward a model that the swelling causes modification of the target cell membrane responsible for the enhancement of phospholipid transfer, although we do not yet disregard the previous model based on the transplanted F proteins. The characteristic kinetic features of the transfer can be explained by this model as well. Envelope fusion causes an increase in the cell membrane permeability, allowing water molecules to enter. When the amount of water reaches a certain threshold or the cell swells to a certain threshold volume, phospholipid transfer to the cell membrane is enhanced. This model can explain most satisfactorily the lack of enhancement in ghosts since no swelling occurs with ghosts which lack the cytoplasmic proteins. The swelling would cause expansion of the cell membrane and the cytoskeleton beneath it. The latter could cause rearrangement of integral proteins in the membrane. The transfer of phospholipid to such expanded lipid bilayer sites may be enhanced.

The envelope fusion is a simple reaction in the sense that its rate constant is a constant, independent of the number of viruses adsorbed on the cell membrane. The phospholipid transfer or exchange, on the other hand, is dependent on the number of adsorbed viruses, being autocatalytically accelerated through modification of the cell membrane by virus via swelling. This modification of cell membrane also facilitates cell to cell fusion. It is known that HVJ causes vigorous fusion of intact erythrocytes but slight fusion of ghosts.

Acknowledgments

We thank Roger Lew for reading the manuscript.

References

Homma, M. (1972) *J. Virol. 9*, 829–835.
Homma, M., & Ohuchi, M. (1973) *J. Virol. 12*, 1457–1465.
Homma, M., Shimizu, K., Shimizu, Y. K., & Ishida, N. (1976) *Virology 71*, 41–47.
Ishida, N., & Homma, M. (1978) *Adv. Virus Res. 23*, 349–383.
Knutton, S. (1976) *Nature (London) 264*, 672–673.
Knutton, S. (1977) *J. Cell Sci. 28*, 189–210.
Knutton, S. (1979) *J. Cell Sci. 36*, 85–96.
Knutton, S., & Bächi, T. (1980) *J. Cell Sci. 42*, 153–167.
Kuroda, K., Maeda, T., & Ohnishi, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 804–807.
Lyles, D. S., & Landsberger, F. R. (1979) *Biochemistry 18*, 5088–5095.
Maeda, T., Asano, A., Ohki, K., Okada, Y., & Ohnishi, S. (1975) *Biochemistry 14*, 3736–3741.
Maeda, T., Asano, A., Okada, Y., & Ohnishi, S. (1977) *J. Virol. 21*, 232–241.
Maeda, T., Ohnishi, S., & Yanagida, M. (1978) *FEBS Lett. 89*, 29–32.
Poste, G., & Nicolson, G. L., Eds. (1977) *Cell Surf. Rev. 2*.
Poste, G., & Nicolson, G. L., Eds. (1978) *Cell Surf. Rev. 5*.
Salk, J. E. (1944) *J. Immunol. 49*, 87–98.
Shimizu, K., & Ishida, K. (1975) *Virology 67*, 427–437.
Steck, T. L. (1974) *Methods Membr. Biol. 2*, 245–281.
Stone, T. J., Buckman, T., Nordio, P. L., & McConnell, H. M. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1010–1017.