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STUDIES ON THE VIRUS-INDUCED FUSION
OF HUMAN ERYTHROCYTE GHOSTS

KIYOTOSHI SEKIGUCHI

I. CHARACTERIZATION OF VIRUS-INDUCED FUSION OF HUMAN ERYTHROCYTE GHOSTS

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SUMMARY

Although human erythrocyte ghosts prepared by hypotonic hemolysis were incapable of undergoing HVJ (Sendai virus)-induced fusion, the fusion activity could be restored by including bovine serum albumin (BSA) within the ghosts. The fusion of BSA-loaded ghosts was similar to that of intact erythrocytes in that both were dependent on the viral F glycoprotein and proceeded in the absence of calcium ion and ATP. The fusion of BSA-loaded ghosts, however, required a viral dose that was 10 times as high as that for the fusion of intact erythrocytes. Freeze-fracture electron microscopy indicated that rearrangement of the intramembrane particles took place during the fusion of both intact cells and BSA-loaded ghosts, though its extent was lesser in the latter. Little changes in the particle distribution was observable on interaction of the virus with non-fusible ghosts lacking internal BSA. Measurement of both virus-induced lysis and phospholipid intermixing between the viral envelope and cell membrane, however, indicated that the efficiency of the viral fusion with the cells ("envelope fusion") was not significantly different between the non-fusible and fusible ghosts. These observations suggested that the rearrangement of intramembrane particles was an essential step of the virus-induced cell-cell fusion. Besides BSA, such macromolecules as ovalbumin and dextran could also restore the fusion activity of the ghosts, although hemoglobin and polyethylene glycol were ineffective. All these macromolecules, when added to the fusion system externally, inhibited the process. They also inhibited the fusion of intact erythrocytes at concentrations that were higher by nearly one order of magnitude than those effective for the fusion of BSA-

loaded ghosts. It was suggested that the concentration gradient of macromolecule across the cell membrane was one of the factors controlling the fusion reaction.

INTRODUCTION

In cells, membrane fusion plays important roles in such cell activities as endocytosis and subsequent intracellular digestion of extracellular materials and secretion of intracellular substances (exocytosis) (1). Membrane fusion is also involved in physiological cell fusion phenomena such as fertilization (2) and differentiation of muscle cells (3) and also in pathological processes such as viral infection (1,4) and inflammation (5). Despite its importance in cell biology, the mechanism of membrane fusion is not yet well understood, partly because of the lack of suitable experimental systems by which the basic molecular events involved can be studied.

Virus-induced cell fusion, first reported by Okada (6), has served as a good experimental model for studies of membrane fusion, because in this system rapid and extensive fusion can be attained under easily controllable conditions. Thus, Okada et al. (7,8) have reported that metabolic energy and calcium ion are required for HVJ (Sendai virus)-induced fusion of Ehrlich ascites tumor cells. Recent studies have further shown that the intracellular level of cyclic AMP increases transiently during the fusion reaction (9) and that cytochalasin D, a potent inhibitor of microfilaments, depresses the fusion efficiency (10). However, little is as yet understood of the structural changes in the cell membrane responsible for the fusion reaction.

To obtain this type of information, the use of human erythrocytes seems to be more advantageous than that of Ehrlich ascites cells, because the erythrocytes are devoid of intracellular organelles and their plasma membrane can be prepared easily by simple hypotonic hemolysis (11). Furthermore, the erythrocyte membrane

is one of the most extensively characterized biomembranes and much is known of its structure and function. For instance, the asymmetric dispositions of its lipid and protein components have been determined (12,13).

Because of these advantages, cell fusion of erythrocytes from several animals has recently been extensively studied (14-36). For example, Peretz et al. (18) have shown that the virus-induced fusion of human erythrocytes requires neither metabolic energy nor calcium ion. Using freeze-fracture electron microscopy, Bächli et al. (16, 17) have reported that the intramembrane particles undergo redistribution in an early stage of virus-induced fusion of human erythrocytes. Similar lateral rearrangement of the intramembrane particles has also been reported to occur in fusion of chicken erythrocytes and human erythrocyte ghosts induced by non-viral fusogenic agents (24,25,30) and in membrane fusion accompanying secretion by mast cells (31-33) and Tetrahymena (34) as well as conjugation of protozoan (35). Since the intramembrane particles observable by freeze-fracture electron microscopy are supposed to represent the hydrophobic segments of glycoproteins which span the membrane lipid bilayer, it is suggested that lateral redistribution of membrane glycoproteins is a structural alteration common to various membrane fusion phenomena.

In the case of human erythrocytes, evidence has been presented that the topological distribution of membrane glycoproteins is regulated by spectrin and probably also by erythrocyte actin, proteins which are associated with the cytoplasmic surface of the membrane, and are assumed to form a meshwork structure (36-40). Therefore, if the rearrangement of membrane glycoproteins is actually a pre-

requisite to the achievement of membrane fusion, it is expected that structural perturbation of the spectrin meshwork will affect the fusion efficiency of human erythrocytes. Such structural perturbation can be relatively easily accomplished using erythrocyte ghosts. However, human erythrocyte ghosts prepared by hypotonic hemolysis have lost their fusion ability, though still retain the activity to be agglutinated by HVJ (27). Only those ghosts prepared by either gradual hemolysis in the presence of albumin (18) or hypotonic (rapid) hemolysis after blocking of membrane sulfhydryl groups (27) can be fused by the virus.

This paper describes a method by which the fusion activity of human erythrocyte ghosts once lost by hypotonic hemolysis can be restored. The properties of the virus-induced fusion of the thus treated ghosts are also reported. This fusion system seems to be very suitable for studies of the structural alterations in the cell membrane during the fusion reaction.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA, fraction V), fatty acid-free bovine serum albumin, ovalbumin, trypsin, soybean trypsin inhibitor, polyvinylpyrrolidone (PVP-40) and Tricine (N-tris[hydroxymethyl]methyl glycine) were obtained from Sigma Chemical Co., dextran T 70 (av. mol.wt. 70,000) was from Pharmacia Fine Chemicals, adenosine 5'-triphosphate disodium salt was from Kohjin Co., glutaraldehyde was from Ladd Research Industries Inc. and Na¹²⁵I (carrier free) was from New England Nuclear Corp. Spin-labeled phosphatidylcholine (PC*) was prepared by reacting egg lysolecithin with 12-nitrooxide stearic acid anhydride by the method of Hubbell and McConnell (41). Other inorganic chemicals were of reagent grade or the highest grade available. A buffer consisting of 135 mM NaCl, 5.4 mM KCl and 40 mM Tricine-NaOH, pH 7.6 was used as the medium throughout and is referred to as TBS (Tricine-buffered saline).

Cells and Virus. Human blood, stored in ACD solution, from donors of different blood types, was obtained from a blood bank (Midorijuji, Osaka) and used within four weeks after drawing. For the fusion experiment of intact erythrocytes, cells were washed four times with PBS (phosphate-buffered saline composed of 0.9 % NaCl and 5 mM sodium phosphate, pH 7.6), twice with TBS and finally suspended in TBS to give a concentration of about 2 % (v/v). HVJ (Sendai virus), Z strain, was propagated in embryonated eggs. The virus was purified from the infected chorioallantoic fluid by two cycles of differential centrifugation (42) and suspended in TBS. The dose of the virus was expressed in terms of its hemagglutination unit (HAU) which was determined by Salk's pattern method (43).

Trypsin digestion of HVJ was carried out according to Shimizu and Ishida (44). HVJ, washed once with 10 mM sodium phosphate, pH 7.2 and resuspended in the same buffer, was incubated with trypsin (25 $\mu\text{g/ml}$) at 37° for 20 min. The digestion was stopped by adding soybean trypsin inhibitor (50 $\mu\text{g/ml}$). The digested virus was washed with TBS twice, and suspended in TBS.

Preparation of Ghosts and Loading of BSA and Other Macromolecules.

Erythrocyte ghosts were prepared according to Dodge et al. (11). Washed erythrocytes were hemolyzed by adding 20 vol of 5 mM sodium phosphate buffer (pH 8.0). The lysed cells were immediately sedimented by centrifugation at 15,000 rpm for 20 min in a Sorvall SS-34 rotor at 4° and washed once with the same buffer. One vol of packed ghosts was mixed with four vol of TBS containing BSA or other macromolecules (loading medium) and allowed to stand for 15 min at 0°, followed by resealing by incubating at 37° for 60 min. Ghosts loaded with BSA or other macromolecules were washed twice with TBS and suspended in TBS to give a concentration of about 2 % (v/v).

Fusion Experiments. Unless otherwise stated, 0.19 ml of 2 % intact cells or ghosts was mixed with 0.01 ml of HVJ and kept in ice for 15 min to allow agglutination. The resulting aggregates were incubated at 37° for 30 min or 60 min and then chilled in ice to terminate the fusion reaction. Fused cells or ghosts were examined by phase contrast microscopy.

Radioiodination of BSA. To a mixture containing Na^{125}I (50 $\mu\text{Ci/ml}$) and 5 % BSA in TBS was added chloramine T (20 $\mu\text{g/ml}$), and the mixture was incubated at 4° for 10 min with gentle shaking. Iodination was then stopped by the addition of sodium metabisulfite

(20 µg/ml). Iodinated BSA was passed through a Sephadex G 50 column to remove low molecular weight compounds. [125 I]BSA thus obtained was concentrated by vacuum dialysis and stored at -20°.

Measurement of Virus-Induced Lysis. For measurement of HVJ-induced hemolysis, intact erythrocytes were agglutinated by HVJ at 0° for 15 min, and the mixture was then incubated at 37° for different periods of time. After the addition of four vol of cold TBS to stop the reaction, the diluted cell suspension was centrifuged at 3,000 rpm for 15 min at 4°. The absorbance of the supernatant at 540 nm was taken as the extent of hemolysis. To determine virus-induced lysis of ghosts, ghosts were loaded with [125 I]BSA. The loaded ghosts were treated in the same way as above, and the amount of [125 I]BSA in the supernatant was measured in a well-type scintillation spectrometer (Aloka Auto Well Gamma System, JDC-751).

Preparation of Spin-Labeled HVJ. Spin-labeled HVJ was prepared as described by Maeda et al. (20). PC* (2 mg/ml) suspended in TBS was sonicated under a stream of nitrogen gas in an ice bath for 10 min and the sonicated mixture was centrifuged at 100,000xg for 60 min at 4°. The supernatant was mixed with an equal vol of HVJ (the molar ratio of the viral lipids to PC* was about 0.2) and incubated at 37° for 6 h. Spin-labeled HVJ was washed with TBS containing BSA (10 mg/ml), then with TBS, and finally suspended in TBS. The resulting HVJ was densely labeled with PC* and the electron resonance (ESR) spectrum of the virus was broadened by spin-spin exchange interaction.

Assay of Phospholipid Intermixing between Viral Envelope and the Erythrocyte Membrane. Intact erythrocytes (or BSA-loaded ghosts) were agglutinated by HVJ (a mixture of HVJ* and non-

labeled HVJ) for 15 min in an ice bath and the agglutinated cells (or ghosts) were collected by centrifugation at 2,000 rpm for 5 min. The packed agglutinated cells (or ghosts) were immediately taken into a quartz capillary tube and ESR spectrum was recorded at 37° on a JEOLCO ME-X spectrometer. Increase in the central peak height was measured and plotted against incubation time.

Freeze-Fracture Electron Microscopy. The samples were fixed by the addition of an equal vol of 4 % glutaraldehyde dissolved in TBS and prewarmed to the temperature of the reaction. Fixation was performed for 40 min at 37° or 2 h at 4° in case of control cell aggregates which was not incubated at 37°. After three washes with TBS, the fixed samples were suspended in TBS containing 20 % glycerol and allowed to stand at 4° overnight. Freeze-fracturing was performed at standard conditions by a Hitachi freeze-fracture apparatus. Electron micrographs were taken by a Hitachi electron microscope.

Measurement of the Number of Ghosts. The number of ghosts was counted with a Coulter Counter Model A (Coulter Electronics Ltd.) fitted with a 100 μ m aperture. The numbers obtained were reproducible within ± 2 % of the mean value.

Assay. Protein was determined by the method of Lowry et al. (45).

RESULTS

Restoration of Fusion Activity of Erythrocyte Ghosts by BSA

Loading. Addition of HVJ to human erythrocytes in the cold caused the binding of the virus to the cell surface and subsequent agglutination of cells. Upon raising the temperature to 37°, the agglutinated cells fused with one another to form "polyerythrocytes", accompanied by the dissociation of the cell aggregates (Fig. 1A). During the fusion reaction, hemolysis also took place to different extents. These observations were in agreement with previous results (14,18,22). Erythrocyte ghosts prepared by the method of Dodge et al. (11), on the other hand, could no longer fuse even with a viral dose that was 10 times as high as that for erythrocytes, though they could still be agglutinated by the virus (Fig. 1C). Moreover, no fusion could be seen even after resealing of the ghosts by incubation in an isotonic buffer at 37°. It was, however, found that the ghosts resealed in the isotonic buffer containing 5 % BSA and washed could fuse effectively in the presence of HVJ (Fig. 1D). It was, therefore, certain that resealed ghosts containing BSA inside had regained the fusion activity of parent erythrocytes. For the fusion to take place, BSA should be present inside the ghosts, and BSA added exogenously was rather inhibitory as described below. The BSA-containing ghosts before the virus treatment remained at the top upon centrifugation in a dextran T 70 linear density gradient, whereas unsealed ghosts moved toward the bottom (Fig. 2). This indicated that the BSA-containing ghosts were completely resealed. The apparent density of the BSA-loaded ghosts was 1.018 and that of control resealed ghosts was 1.012. The small difference in density was probably due to the BSA present in the former.

The amount of BSA taken up by resealed ghosts increased as the BSA concentration in the resealing medium was increased (Fig. 3), whereas the fusion activity of the ghosts was partially restored with 3 % BSA and maximally with 5 % BSA (Fig. 4). Further increase in BSA concentration in the resealing medium impaired the integrity of the cell membrane after HVJ treatment, as evidenced by vesiculation of the sample (Fig. 4F), and thus decreased the fusion activity.

Participation of F Glycoprotein in the Fusion Reaction of BSA-Loaded Ghosts. The biological activities of HVJ are associated with two glycoproteins which form distinct "spikes" on the viral envelope (46). Hemagglutination and neuraminidase activities are associated with one glycoprotein, HANA, whereas hemolytic and cell fusion-inducing activities are associated with the other glycoprotein, F. Selective cleavage of F glycoprotein by mild digestion with trypsin (44) results in the loss of the hemolytic and cell fusion-inducing activities of the virus, though it can still agglutinate erythrocytes. Fig. 5 shows that trypsinized HVJ could no longer induce the fusion of intact erythrocytes and BSA-loaded ghosts, confirming that the fusion of BSA-loaded ghosts is dependent on F glycoprotein and therefore it can be a prototype of other virus-induced cell fusion reaction.

Kinetic Properties. Fig. 6 shows the time course of fusion of BSA-loaded ghosts and intact erythrocytes. After completion of virus-induced agglutination at 0° for 15 min (Fig. 6A and F), the aggregates were transferred to 37° and extent of fusion was examined as a function of incubation time. Both intact cells and BSA-loaded ghosts were still agglutinated and only a small number of fused cells

and ghosts could be seen after 5 min of incubation (Fig. B and G). On further incubation fusion of the cells and ghosts and partial lysis became more evident, concomitant with dissociation of the aggregates (Fig. C-E and H-J). Upon prolonged incubation the cells and ghosts became swollen and fused ghosts looked like large smooth balloons. This was probably due to colloidal osmotic swelling caused by changes in membrane rigidity induced by fusion between virus envelope and cell membrane. There was no significant difference in time course between fusion of intact cells and ghosts and after 30-60 min of incubation the fusion reaction was almost complete. Further prolonged incubation at 37° resulted in extensive lysis and disintegration of the cell membrane and therefore incubation time at 37° of fusion experiment was fixed 30 min for intact cells and 60 min for BSA-loaded ghosts unless otherwise stated.

Figs. 7 and 8 show the dependency of the cell fusion on the viral dose. As little as 50 HAU/ml of HVJ could induce fusion of intact erythrocytes, though only to a limited extent, and by increasing the viral dose extensive fusion could be obtained (Fig. 7). Corresponding to the increase of the extent of cell fusion, lysis was also accelerated and the number of non-lysed cells which appeared as small bright rings was decreased. When more than 1,000 HAU/ml of HVJ was applied to intact cells, rather extensive lysis and disintegration of the fused cells took place.

When BSA-loaded ghosts were treated with the same amount of HVJ that could cause maximal fusion of intact erythrocytes, however, only a slight fusion could be observed (Fig. 8A). Optimal dose for fusion of BSA-loaded ghosts was between 3,000-6,000 HAU/ml, which was ten times as much as that for intact cells, and increase

in viral dose sometimes resulted in impairment or disintegration of cell membrane (Fig. 8B-F). This discrepancy in viral doses for maximal fusion between intact cells and BSA-loaded ghosts may reflect the alteration in membrane integrity which was caused by hypotonic hemolysis.

Fig. 9 shows the temperature dependency of fusion reaction of both intact cells and BSA-loaded ghosts. Fusion of intact cells was significantly diminished by lowering temperature to 30°. Below 25° no fusion could be observed and cells remained agglutinated even after incubation for 30 min. On the other hand, fusion of BSA-loaded ghosts was not significantly inhibited at 30° and some extent of fusion was observed at 25°. It was completely inhibited below 25° whereas agglutinated ghosts were almost dissociated in spite of complete inhibition of fusion reaction.

Effect of Calcium Ion and Internal ATP. Although the fusion of several nucleated cells has been shown to require calcium ion and metabolic energy (7,8), no such requirements have been reported for the fusion of human erythrocytes (18). The fusion of BSA-loaded ghosts could also proceed in the absence of calcium ion and internal ATP. As shown in Fig. 10B and C, no stimulation of the fusion efficiency was observed when calcium ion was included inside the BSA-loaded ghosts; calcium ion rather caused slight inhibition. Fig. 10D shows that internal ATP was also ineffective in promoting the fusion of BSA-loaded ghosts. This indicates that ATP is not required for the fusion reaction as a source of metabolic energy or a phosphate donor for membrane phosphorylation, although it has been reported that incubation of ghosts with MgATP at 37° resulted in phosphorylation of spectrin (47).

Virus-Induced Lysis of Intact Erythrocytes and Their Ghosts. Interaction of intact erythrocytes with HVJ resulted in swelling of the cells and eventually in their lysis. Several lines of evidence indicate that the fusion of the viral envelope with the cell membrane ("envelope fusion") causes alterations in the structural integrity of the cell membrane leading to lysis of the cells. The virus-induced lysis of the cells may, therefore, be taken as a measure of envelope fusion. By using this criterion it was examined if envelope fusion was involved in the fusion of BSA-loaded ghosts. As clearly shown in Fig. 11, leakage of BSA took place during incubation with HVJ at 37° and the time course of leakage closely resembled that of virus-induced release of hemoglobin from intact erythrocytes. The resealed ghosts, which contained only a small amount of BSA and therefore could not fuse, also released the internal BSA to almost the same extent as the ghosts sufficiently loaded with BSA. The level of leakage of BSA from ghosts was somewhat lower than that of hemolysis, even though nearly ten times as much as HVJ was used in the former case. These observations indicate that the efficiency of envelope fusion of ghosts was not affected by the amount of BSA loaded but it was significantly reduced compared with that of intact erythrocytes.

To confirm these observations, envelope fusion was also examined by the spin label technique (20,21). This method measures a decrease in the exchange broadening of electron spin resonance (ESR) spectrum when densely labeled membranes are mixed with unlabeled membranes. To evaluate the extent of envelope fusion, HVJ envelope was densely labeled with spin-labeled phosphatidylcholine (PC*) and dilution of PC* during virus-induced cell fusion was

followed. The results thus obtained are shown in Fig. 12. The pattern of disappearance of exchange broadening, which was expressed by the increase of relative peak height, was quite similar to that of virus-induced lysis of cells or ghosts, confirming the conclusion derived from the measurement of virus-induced lysis.

Changes of Lateral Distribution of Intramembrane Particles during Cell Fusion. In the last few years, several lines of evidence have accumulated suggesting that the rearrangement of intramembrane particles takes place at an early stage of membrane fusion. Bächli et al. showed that aggregation of intramembrane particles could be observed when human erythrocytes, which were agglutinated by HVJ at 0°, were incubated at 37° for 2 min and it became dominant after 10 min (17). Fig. 13 confirms their observation. Although intramembrane particles were still randomly distributed after agglutination of erythrocytes by HVJ at 0° (Fig. 13B), subsequent incubation at 37° induced aggregation of the particles (Fig. 13 C). It should be pointed out that pits on the external fracture face, which may represent the place where the particles were pulled out, were found to be also aggregated corresponding to the distribution of the particles seen on the protoplasmic fracture face. When BSA-loaded ghosts were fused by HVJ, similar rearrangement of the particles were observed as seen in Fig. 14B. On the other hand, little or no redistribution of the particles could be observed when ghosts, which were resealed in the absence of BSA, were treated with the same amount of HVJ (Fig. 14A). The close correlation between fusion ability of ghosts and virus-induced rearrangement of intramembrane particles indicates that redistribution of the particles may be a prerequisite for the fusion between two

closely apposed cell membranes.

Restoration of Fusion Ability of Ghosts by Inclusion of Other Macromolecules. To elucidate the role of included BSA in restoration of fusion ability of ghosts, several other macromolecules were loaded in the ghosts and the fusion ability of these ghosts was examined. Loading of defatted BSA also restored the fusion ability of ghosts, though to somewhat lesser extents (Fig. 15B). Inclusion of ovalbumin resulted in the restoration of a rather weak fusion ability of ghosts (Fig. 15C). Not only proteins but also polysaccharides such as dextran could restore fusion ability of ghosts, as shown in Fig. 15D, although the concentration of dextran required for maximal restoration was rather high (20-30 %) compared with BSA. Unexpectedly, however, hemoglobin failed to restore the fusion ability at any concentrations added (Fig. 15E). Furthermore, synthetic macromolecules such as polyvinylpyrrolidone (av. mol. wt. 40,000) and polyethylene glycol (av. mol. wt. 20,000) could restore no or little, if any, fusion ability of ghosts. The role of included macromolecules in restoration of the fusion ability, therefore, could not be easily evaluated, but one possible factor, though it can not explain the mechanism of restoration of fusion ability by itself, is the osmotic gradient produced by loading macromolecules within ghosts.

Inhibition of Virus-Induced Fusion by Externally Added Macromolecules.

Fig. 16 shows the effect of externally added BSA on fusion reaction of BSA-loaded ghosts. After ghosts were agglutinated by HVJ at 0° for 15 min, BSA was added to reaction medium to give concentrations as indicated and immediately transferred to 37°. While fusion reaction was not significantly affected by less

than 0.5 % of BSA, it was critically inhibited by 1-2 % BSA, which must have caused a significant reduction of the colloid osmotic gradient. Similar results could be obtained by other macromolecules added to the reaction medium. Fig. 17 shows that dextran T 70 (\bar{M}_n , 40,300) and polyvinylpyrrolidone (PVP-40, av. mol. wt. 40,000) could also suppress the virus-induced fusion of BSA-loaded ghosts, and furthermore the inhibitory concentrations were almost the same as that of BSA. Hemoglobin and polyethylene glycol could inhibit the fusion reaction (data not shown).

To verify the role of possible osmotic effect in the cell fusion reaction, the effect of externally added macromolecules on fusion of intact erythrocytes was also examined. As shown in Fig. 18, 5-10 % of dextran and PVP-40 could almost completely inhibit the fusion reaction, where extensive inhibition of hemolysis also occurred. BSA could also inhibit, though to a lesser extent, the fusion of intact cells with increasing concentrations up to 20 %. It should be pointed out that the concentration needed for maximal inhibition of fusion of intact cells was about an order higher than that required for the inhibition of fusion of BSA-loaded ghosts, being in good agreement with the difference in internal protein concentration between intact cells and the ghosts (51). These results strongly suggest that colloidal osmotic gradient plays some part in virus-induced cell fusion reaction.

DISCUSSION

Peretz et al. (18) reported that human erythrocyte ghosts prepared by the rapid hemolysis method of Dodge et al. (11) had lost their fusion potentiality, which was however retained by those prepared by gradual hemolysis, *i.e.*, dialysis of intact cells against 40 mM NaCl-10 mM Tricine-NaOH (pH 7.4) containing 3 % BSA. They assumed that rapid hemolysis led to the leakage of a membrane constituent(s) required for the fusion process, and suggested that BSA added in the dialysis medium prevented this leakage. Zakai et al. (25,26) found that the ghosts prepared by gradual hemolysis in the absence of BSA could be fused by precipitates of nascent calcium phosphate and this fusion was accompanied by rearrangement of the intramembrane particles into clusters. They proposed that the calcium phosphate precipitates formed inside the ghosts partially extracted the membrane peripheral proteins, spectrin and actin, and thus increased the mobility of the particles. The calcium phosphate precipitates, however, caused significant damages on the cell membrane and disintegration of the fused ghosts occurred after the completion of the fusion process. More recently, Lalazar et al. (27) reported that even the erythrocyte ghosts prepared by rapid hemolysis could be fused with HVJ when they were pretreated with sulfhydryl-blocking reagents. Their lytic conditions were, however, relatively mild, because the lysing buffer contained 30 mM NaCl. It is, therefore, likely that the ghosts thus obtained were resealed immediately after the lysis and contained significant amounts of hemoglobin and cytosolic constituents. In fact, they observed virus-induced leakage of hemoglobin during the fusion reaction. As to the requirement of sulfhydryl-blocking reagents, they suggested

that these reagents modify membrane proteins, especially spectrin, and thus perturb the protein-lipid interactions resulting in an increase in membrane fluidity.

This paper reports that the fusion activity of erythrocyte ghosts prepared by rapid hemolysis can be restored by loading BSA within the resealed ghosts. This finding is inconsistent with the view postulated by Peretz et al. (18) that the loss of fusion activity is due to the leakage of an essential membrane constituent(s). In any way, BSA-loaded ghosts have several advantages over the three systems so far developed for studies of the mechanism of virus-induced cell fusion in the following respects. (1) Fusion of BSA-loaded ghosts closely resembles that of intact cells in dependency of fusion reaction on F glycoprotein, kinetic properties, ineffectiveness of calcium ion and internal ATP, virus-induced lysis and rearrangement of intramembrane particles during fusion process, and inhibition of fusion by external macromolecules. These similarities ensure that fusion of BSA-loaded ghosts can be a good model for the study of the virus-induced cell fusion reaction. (2) Since the ghosts used in the present study are completely free from endogenous soluble components of erythrocytes and more homogeneous than the partially hemolysed samples, this system is simpler than the ghosts prepared by gradual hemolysis. (3) It is possible to include hardly penetrating compounds into BSA-loaded ghosts to test the effect of these compounds on membrane components located on the inner surface of the ghost membrane. As will be reported in the accompanying paper, it has been possible to include antispectrin antibodies into BSA-loaded ghosts and examine their effect on spectrin in relation to the cell fusion mechanism.

Although the general feature of fusion of BSA-loaded ghosts resembles closely that of intact erythrocytes as described above, the fusion activity of the ghosts is considerably lower than that of intact cells. Thus, the fusion of BSA-loaded ghosts requires a dose of the virus which is 10 times as high as that required for the fusion of intact erythrocytes. This suggests that much more viral particles are necessary to induced the structural changes in the ghosts which allow closely apposed membranes to fuse with each other. It is likely that the efficiency of the envelope fusion is also reduced in BSA-loaded ghosts, because the extent of virus-induced lysis and the rate of disappearance of exchange broadening of ESR spectrum of densely spin-labeled virus, two markers of the envelope fusion, are also decreased in BSA-loaded ghosts. Furthermore, the rearrangement of intramembrane particles observable during the fusion reaction is less evident in BSA-loaded ghosts than in intact cells. While homogeneous clustering of intramembrane particles can be seen all over the fracture face of fused intact cells, only local rearrangement of the particles is observable in the case of the virus-treated BSA-loaded ghosts. These reduced susceptibility of BSA-loaded ghosts to the viral actions may be due to the decreased integrity of the cell membrane and partial leakage of membrane components during hypotonic hemolysis.

Ineffectiveness of Calcium Ion and Internal ATP. It has been established that virus-induced fusion of human erythrocytes does not require externally added calcium ion (18). The present study shows further that inclusion of calcium ion within ghosts is not stimulatory but rather slightly inhibitory for the fusion of BSA-loaded ghosts, and the ghosts loaded with both EDTA and BSA possess a

limited fusion activity (data not shown). These observations indicate that internal calcium ion is also unnecessary for the fusion process or a small amount calcium ion which is tightly bound to the erythrocyte membrane and can not be removed by hypotonic lysis and subsequent wash is sufficient to effect the virus-induced fusion of ghosts.

The demonstration of the fusion of BSA-loaded ghosts containing no internal ATP confirms the previous reports that ATP is not required for the fusion of human erythrocytes (18). However, it should be noted that ATP acts not only as an energy source but also as a phosphate donor in cells. Thus Birchmeier and Singer (47) have shown that the shape change of erythrocyte ghosts inducible by MgATP is related to the phosphorylation of spectrin. Pinder et al. (52) have recently reported that the gelation of a mixture of spectrin and erythrocyte actin can occur only when spectrin is phosphorylated. A likely possibility is that the inclusion of MgATP within ghosts would affect the fusion activity of BSA-loaded ghosts. The results obtained in this study have, however, shown that this is not the case.

Role of Rearrangement of Intramembrane Particles in Virus-Induced Cell Fusion. Redistribution of intramembrane particles has been observed not only in the virus-induced fusion of human erythrocytes (16,17) but also in the calcium-induced fusion of chicken and human erythrocytes (24-26,30). Similar changes in the distribution of the particles have also been demonstrated in other membrane fusion phenomena (31-35). On the basis of these observations, it has been proposed that membrane fusion takes place at the region where the particles are excluded and bare lipid bilayer is exposed

(53,54). The finding reported in this paper that HVJ induces the rearrangement of the particles in the membrane of fusible BSA-loaded ghosts but not in the membrane of non-fusible ghosts supports this hypothesis. Further confirmation of the hypothesis will be presented in the accompanying paper, in which detailed molecular mechanisms of erythrocyte ghost fusion are discussed in connection with the role of spectrin in the regulation of mobility of membrane glycoproteins.

The Role of Included Macromolecules in Virus-Induced Cell Fusion Reaction. It is notable that interaction of intact erythrocytes or BSA-loaded ghosts with HVJ always results in the swelling and subsequent lysis of cells or ghosts irrespective of the occurrence of cell fusion. The swelling of cells or the ghosts may be due to a colloidal osmotic gradient across the cell membrane (46) which is produced by included macromolecules and also to the loosening of the spectrin meshwork, which is thought to be responsible for the cell shape and viscoelastic properties of erythrocytes (55-58). The spectrin meshwork may play a more critical part in the swelling of BSA-loaded ghosts because these ghost per se do not swell in spite of the fact that BSA is included only inside the ghosts and the ionic circumstance of both sides of the cell membrane is almost the same. It may be because osmotic gradient produced by included BSA is too weak to overcome the constraints caused by rather native spectrin meshwork to maintain the cell shape. An increase in surface free energy caused by swelling of cells and ghosts may be a thermodynamic prerequisite to the cell fusion reaction. This idea seems to be supported by the fact that inclusion of not only BSA but some other macromolecules restores the fusion activity of the ghosts and furthermore externally added

macromolecules inhibits the fusion of both intact erythrocytes and their ghosts. Recently, Miller and Racker reported that calcium ion-induced fusion of proteoliposomes was significantly promoted by applying a positive osmotic gradient across the liposomal membrane, suggesting that the thermodynamic driving force for the calcium-induced fusion is an excess surface free energy which can be supplied by membrane curvature or transmembrane osmotic gradients (59).

Possible Mechanism of Virus-Induced Fusion of Erythrocytes and Their Ghosts. The role of an osmotic gradient across the cell membrane in the fusion reaction, together with the rearrangement of intramembrane particles during the cell fusion, lead to a possible mechanism for the virus-induced fusion of intact erythrocytes and their ghosts. The adsorption of viral particles onto the cell surface at 0° causes cell agglutination so that two distinct cell membranes are brought into close contact. Subsequent incubation at 37°, fusion between the cell membrane and viral envelope (envelope fusion) occurs and the viral envelope is integrated into cell membrane. The envelope fusion induces structural alteration of the cell membrane which results in an increase in ion permeability of the cell membrane, causing colloidal osmotic swelling and subsequent lysis. It may also perturbs the interaction between the spectrin meshwork and the cytoplasmic surface of the membrane and loosens the spectrin meshwork. The mobility of intramembrane particles is thus increased resulting in the clustering of the intramembrane particles (38). In case of BSA-loaded ghosts, the latter possibility may play a major role as described above. When rearrangement of the particles, which exposes bare lipid bilayer, is induced

on both closely apposed cell membranes, cell-cell fusion can be achieved between the region of bare lipid bilayer, by utilizing excess free surface energy derived from the osmotic swelling.

Recently, Papahadjopoulos et al. proposed that isothermic phase changes of lipid bilayer can be a prerequisite to the fusion of multilamellar liposomes (60-61), though it has not yet been examined whether such phase changes do occur during the virus-induced cell fusion reaction. It is, however, more likely that the envelope fusion perturbs the interaction between the cytoplasmic surface of the erythrocyte membrane and the spectrin-actin meshwork to induce not only the clustering of intramembrane particles but also local phase changes of lipid matrix and these two effects, together with osmotic swelling, allow the agglutinated cells to fuse.

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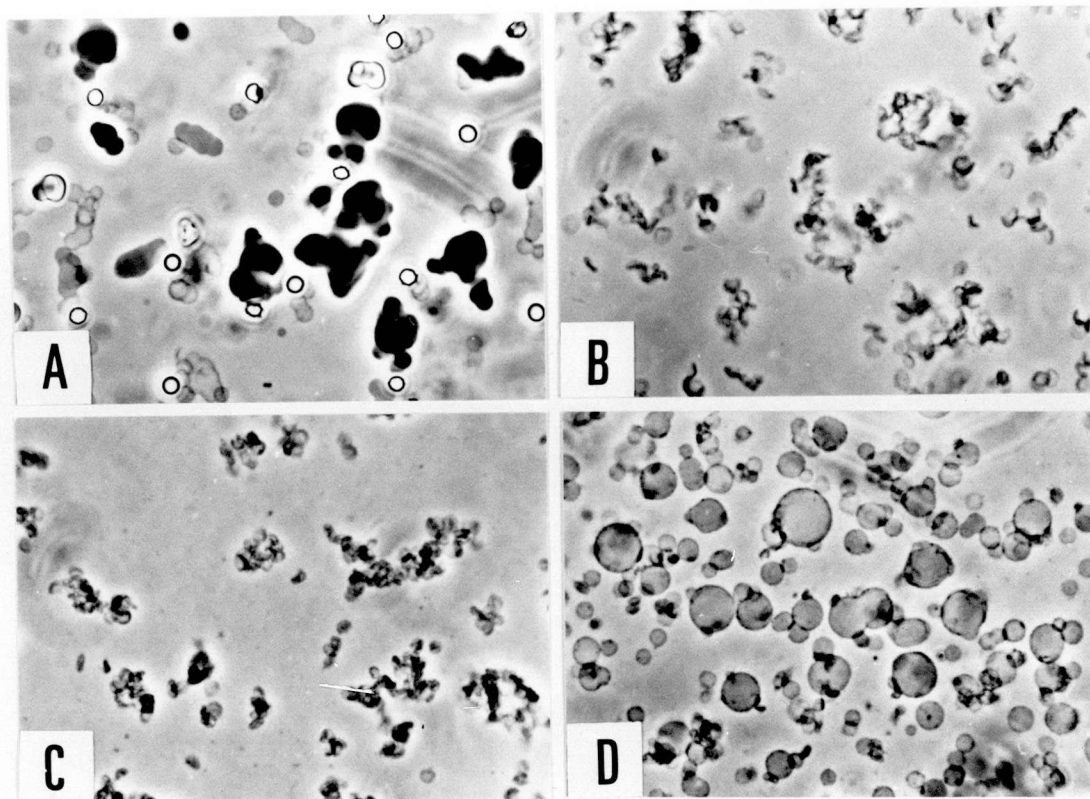


Fig. 1. Restoration of fusion activity of erythrocyte ghosts by including bovine serum albumin. (A), Virus-induced fusion of intact erythrocytes. Intact cells were agglutinated by HVJ (312 HAU/ml) for 15 min in an ice bath, and subsequently incubated for 30 min at 37°. Many large fused cells which partially lost internal hemoglobin could be seen. (B), Ghosts prepared according to Dodge et al (11) and then suspended in TBS were treated with HVJ (312 HAU/ml) as described in (A). Note that no fused ghosts could be observed and ghosts were still somewhat agglutinated even after the end of incubation at 37°. (C), The same as (B) except that applied dose of HVJ was 3,000 HAU/ml. Neither fused ghosts could be seen. (D), The same as (C) except that ghosts loaded with 5 % BSA (see "Materials and Methods") were substituted for unsealed ghosts. Many fused ghosts could be seen. Phase microscopy, x350.

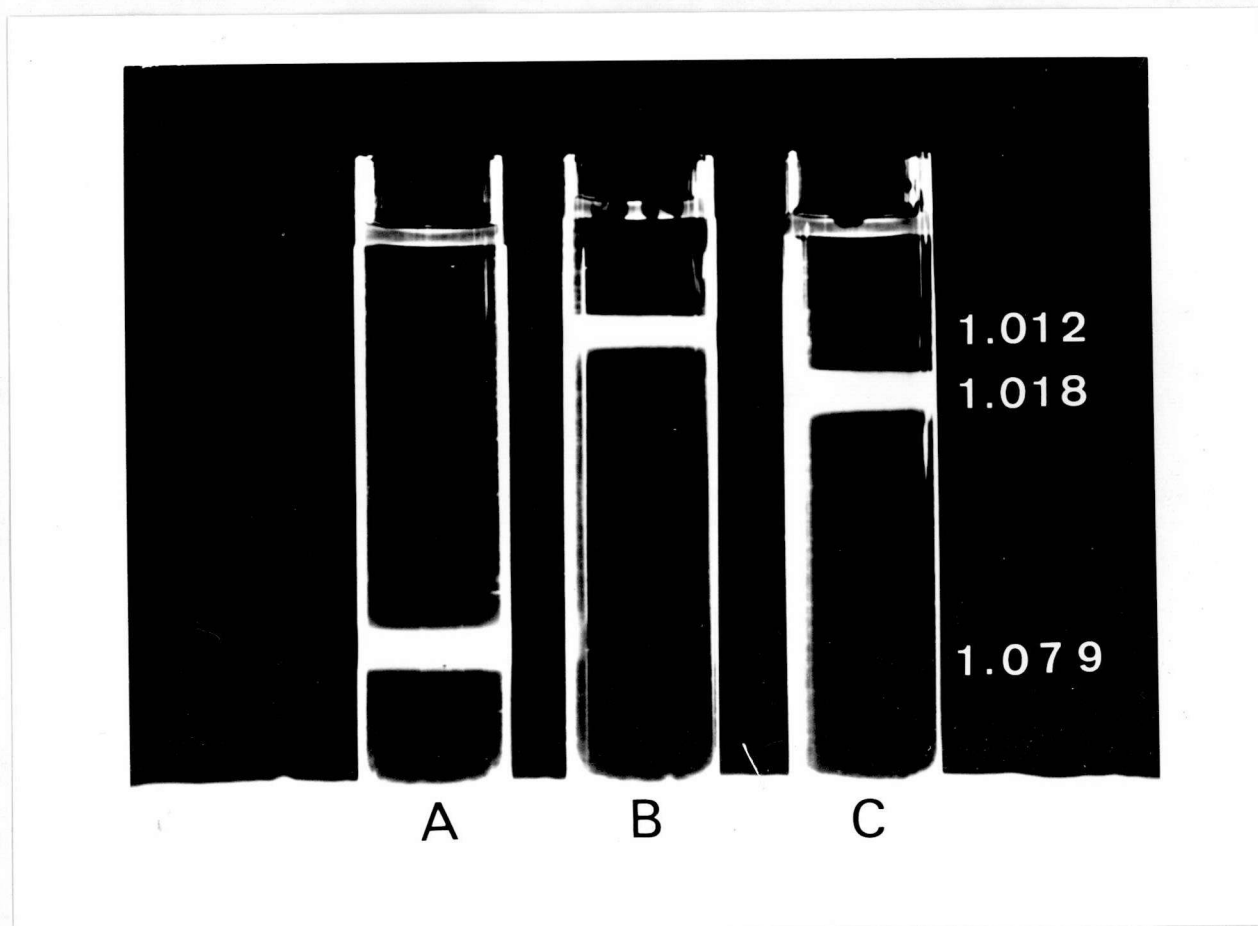


Fig. 2. Dextran T 70 continuous density gradient centrifugation. Continuous linear gradient of dextran T 70 (0-25 %, dissolved in TBS) was formed in Spinco SW 41 rotor tubes. Samples (1 ml) applied on the gradient were (A), unsealed ghosts; (B), ghosts resealed in the absence of BSA; (C), ghosts loaded with 5 % BSA. Samples were centrifuged for 4 h at 40,000 rpm (Spinco SW 41 rotor) at 4°.

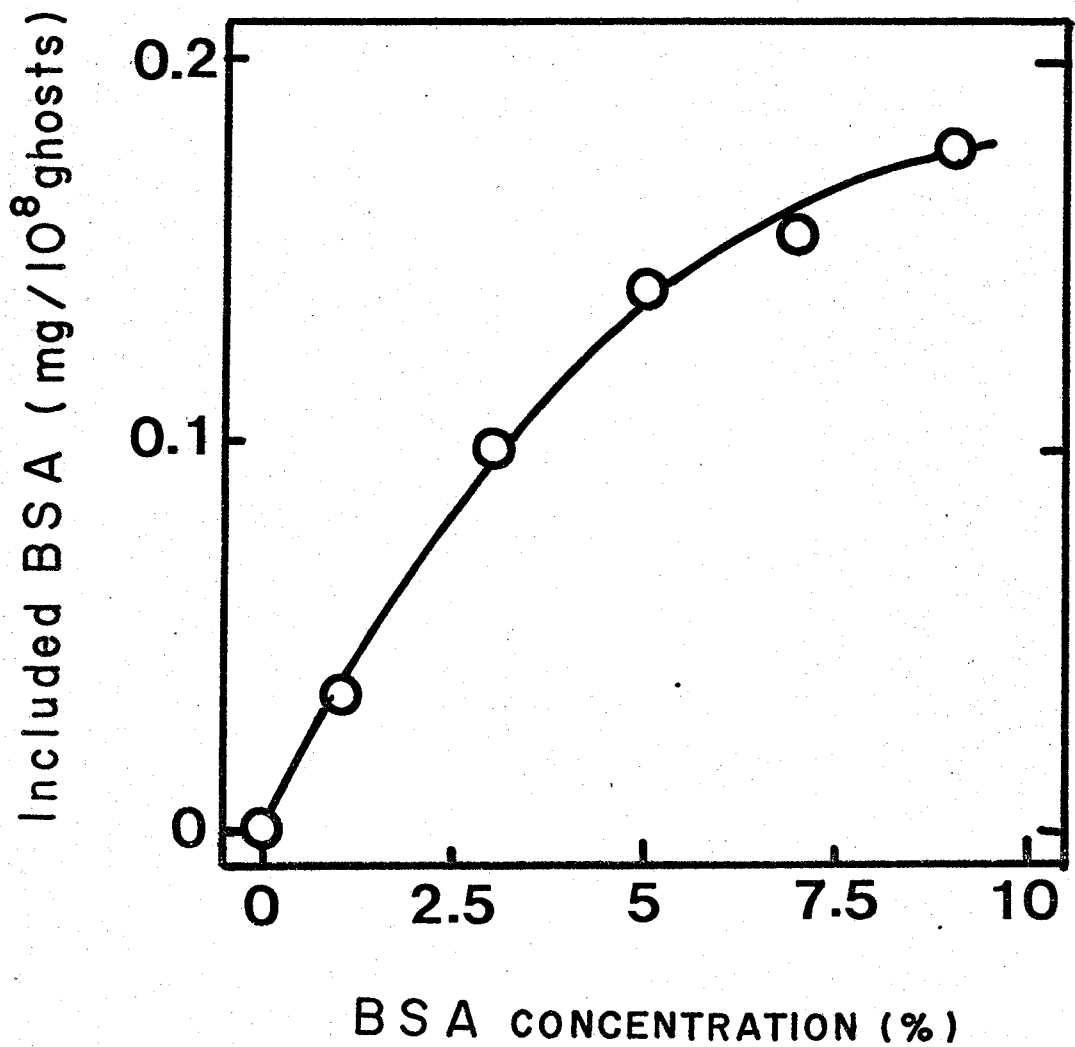


Fig. 3. The amount of BSA included within ghosts. Ghosts loaded with increasing concentrations of BSA were prepared as described in "Materials and Methods". The amount of included BSA was estimated by subtracting the amount of original membrane proteins (which was determined using the ghosts resealed in the absence of BSA) from the total proteins of ghosts. The value was normalized against ghost: number which was determined with a Coulter counter.

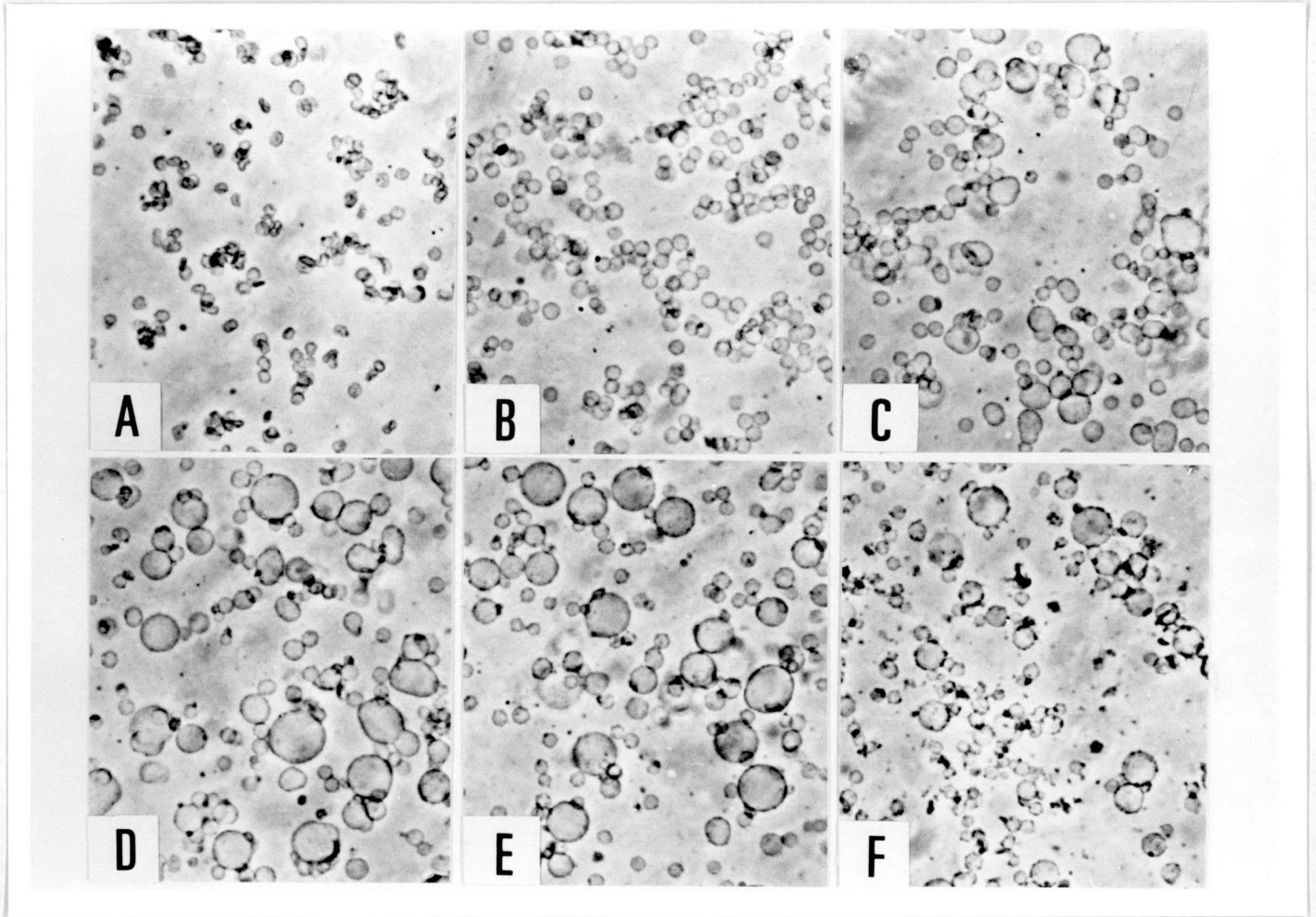


Fig. 4. Dependency of the restored fusion activity of erythrocyte ghosts on the concentration of BSA. Ghosts loaded with BSA of different concentrations were prepared as described in "Materials and Methods". These BSA-loaded ghosts were agglutinated by HVJ (10,000 HAU/ml) for 15 min in an ice bath and subsequently incubated for 30 min at 37°. (A), 0 %; (B), 1 %; (C), 3 %; (D), 5 %; (E), 7 %; (F), 9 %. Phase microscopy, x350.

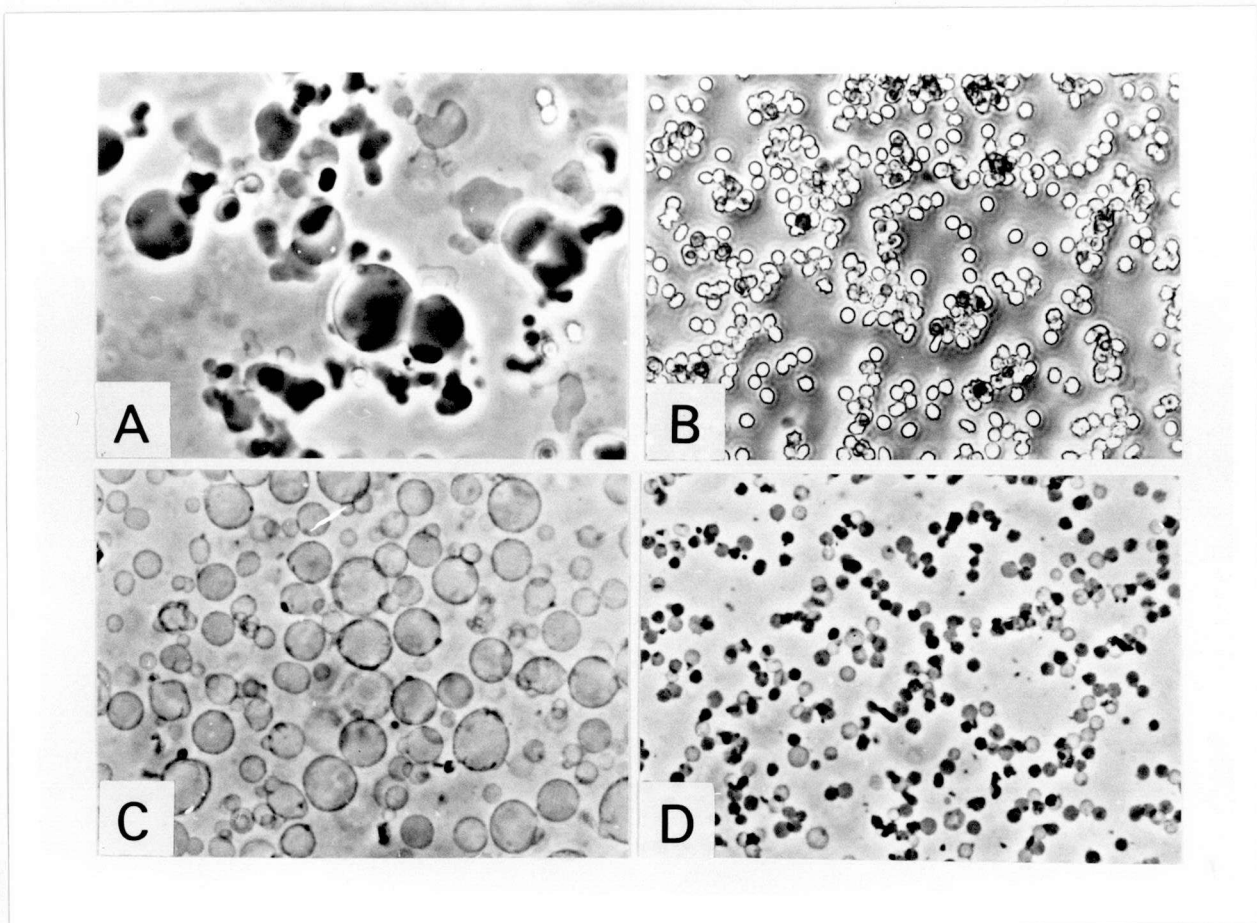


Fig. 5. Dependency of the fusion reaction of BSA-loaded ghosts on viral F glycoprotein. Intact erythrocytes or BSA-loaded ghosts were agglutinated by HVJ (375 HAU/ml for intact cells or 6,000 HAU/ml for the ghosts) for 15 min in an ice bath, then incubated at 37° for 30 min (intact cells) or 60 min (the ghosts). (A), Intact erythrocytes fused by native HVJ; (B), the same as (A) except that trypsinized HVJ was substituted for native virus; (C), BSA-loaded ghosts fused by native HVJ; (D), the same as (C) except that trypsinized virus was used instead of native one. Phase microscopy, x350.

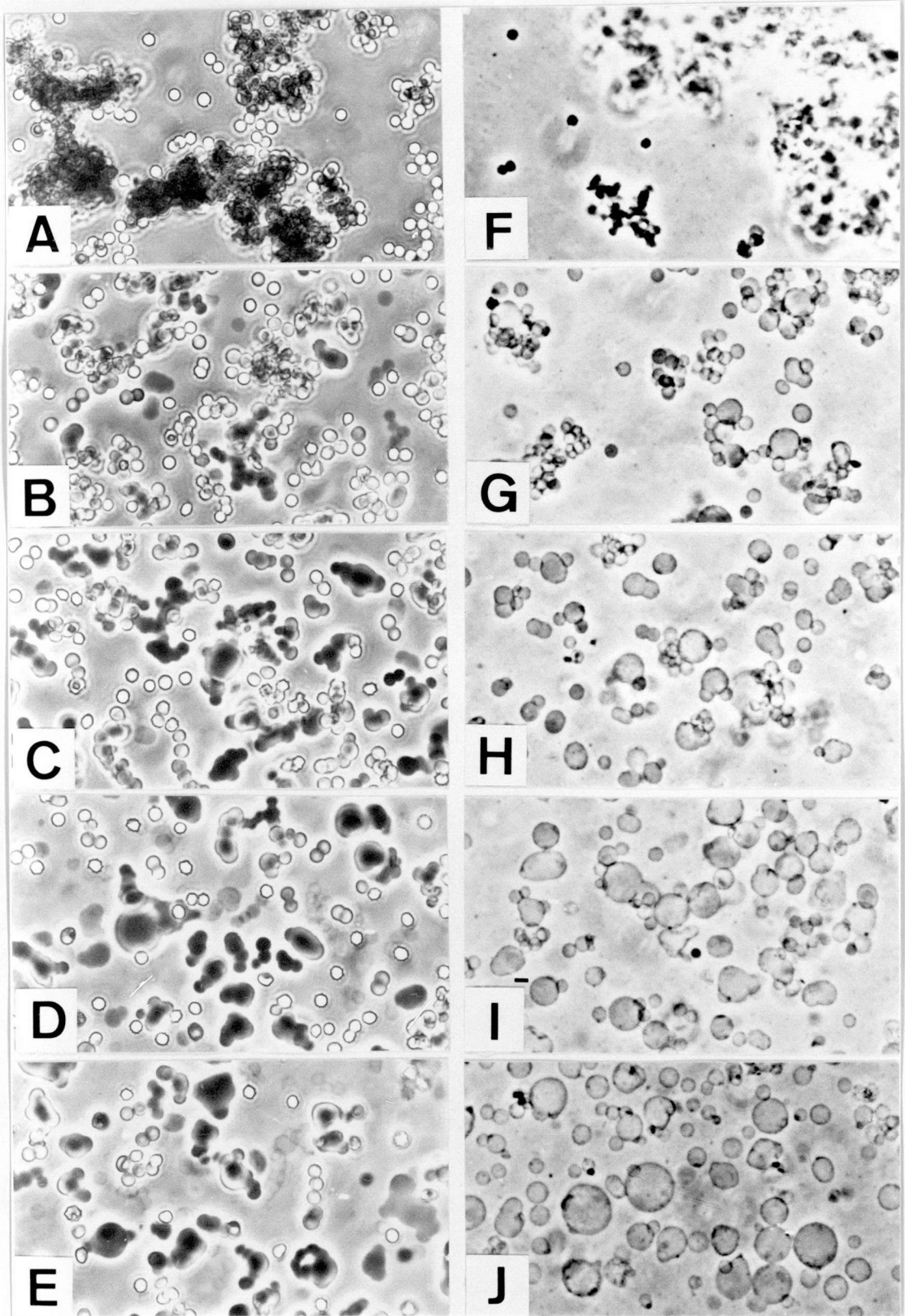


Fig. 6. Time course of the fusion reaction of intact erythrocytes and BSA-loaded ghosts. Intact cells (A-E) and BSA-loaded ghosts (F-J) were agglutinated by HVJ (188 HAU/ml for intact cells and 6,000 HAU/ml for the ghosts) for 15 min at 0°, then incubated at 37° for different periods. (A and F), 0 min; (B and G), 5 min; (C and H), 10 min; (D and I), 30 min; (E and J), 60 min. Phase microscopy, x350.

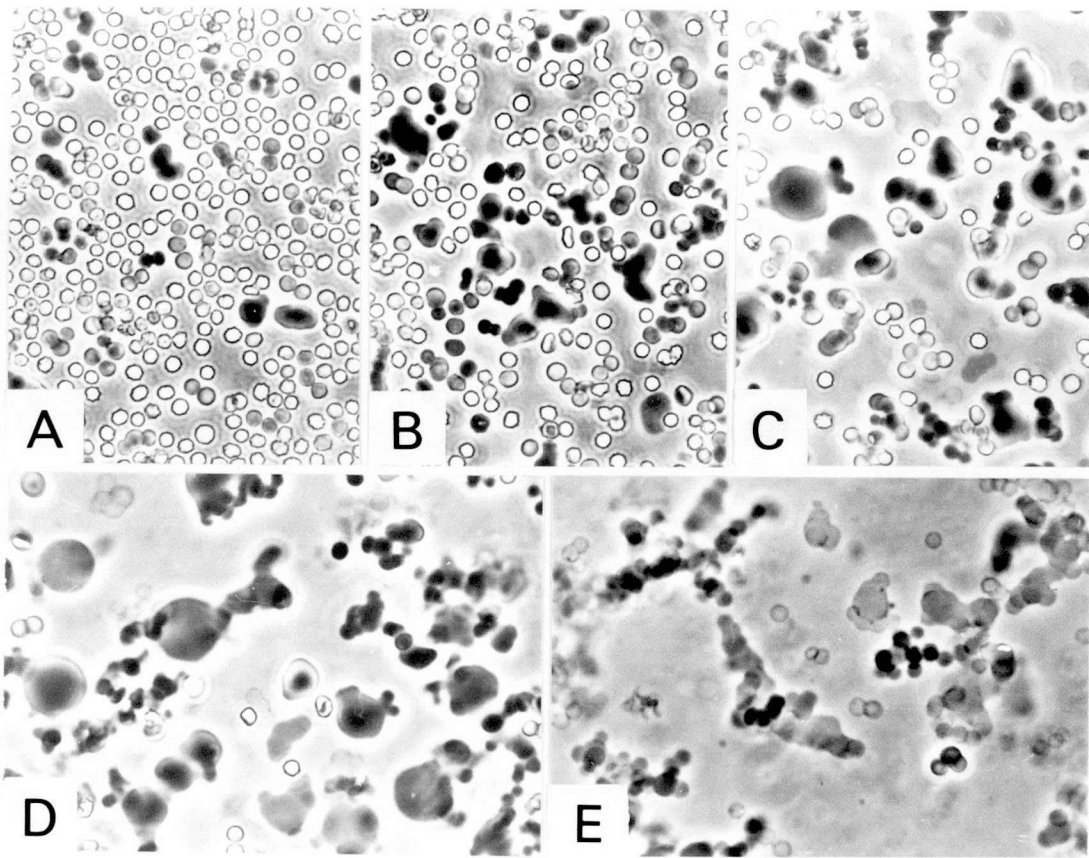


Fig. 7. Dependency of the fusion reaction of intact erythrocytes on viral dose. Intact cells were agglutinated by different doses of HVJ for 15 min in an ice bath and subsequently incubated at 37° for 30 min. (A), 50 HAU/ml; (B), 100 HAU/ml; (C), 188 HAU/ml; (D), 375 HAU/ml; (E), 750 HAU/ml. phase microscopy, x350.

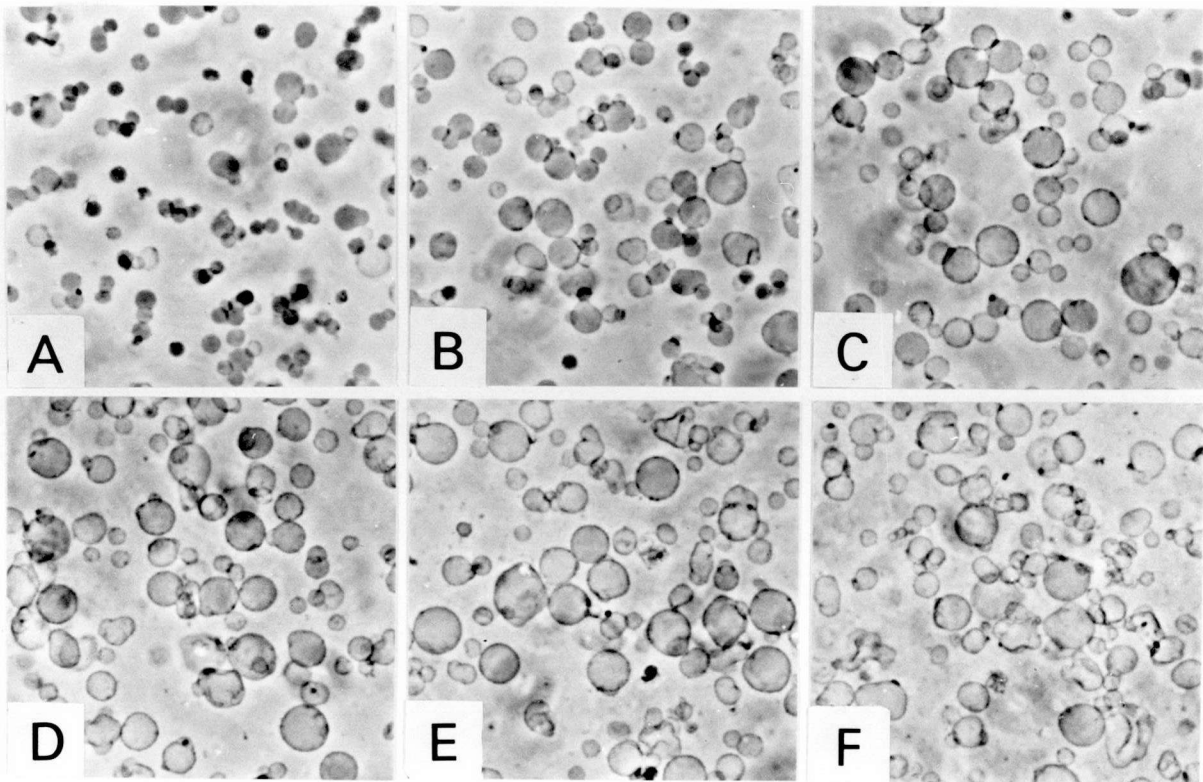


Fig. 8. Dependency of the fusion reaction of BSA-loaded ghosts on viral dose. BSA(5 %)-loaded ghosts were agglutinated by different doses of HVJ for 15 min in an ice bath and subsequently incubated at 37° for 60 min. (A), 375 HAU/ml; (B), 750 HAU/ml; (C), 1,500 HAU/ml; (D), 3,000 HAU/ml; (E), 6,000 HAU/ml; (F), 12,000 HAU/ml. Phase microscopy, x350.

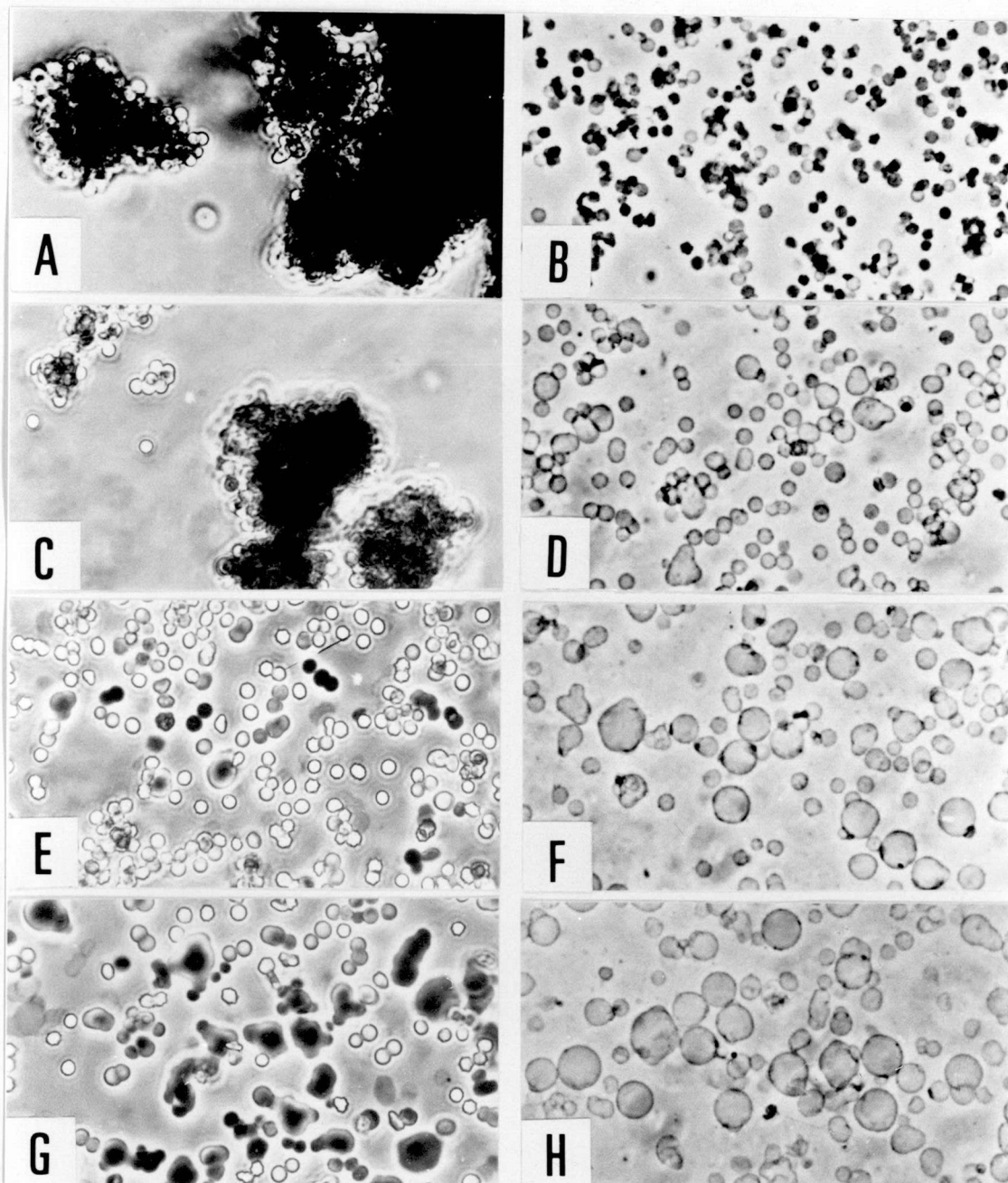


Fig. 9 . Temperature dependency of the fusion reaction of intact erythrocytes and BSA-loaded ghosts. Intact cells (A,C,E,G) or BSA-loaded ghosts (B,D,F,H) were agglutinated by HVJ (188 HAU/ml for intact cells and 6,000 HAU/ml for the ghosts) for 15 min in an ice bath and then incubated at different temperatures for 30 min (intact cells) or 60 min (the ghosts). (A and B), 20°; (C and D), 25°; (E and F), 30°; (G and H), 37°. Phase microscopy, x350.

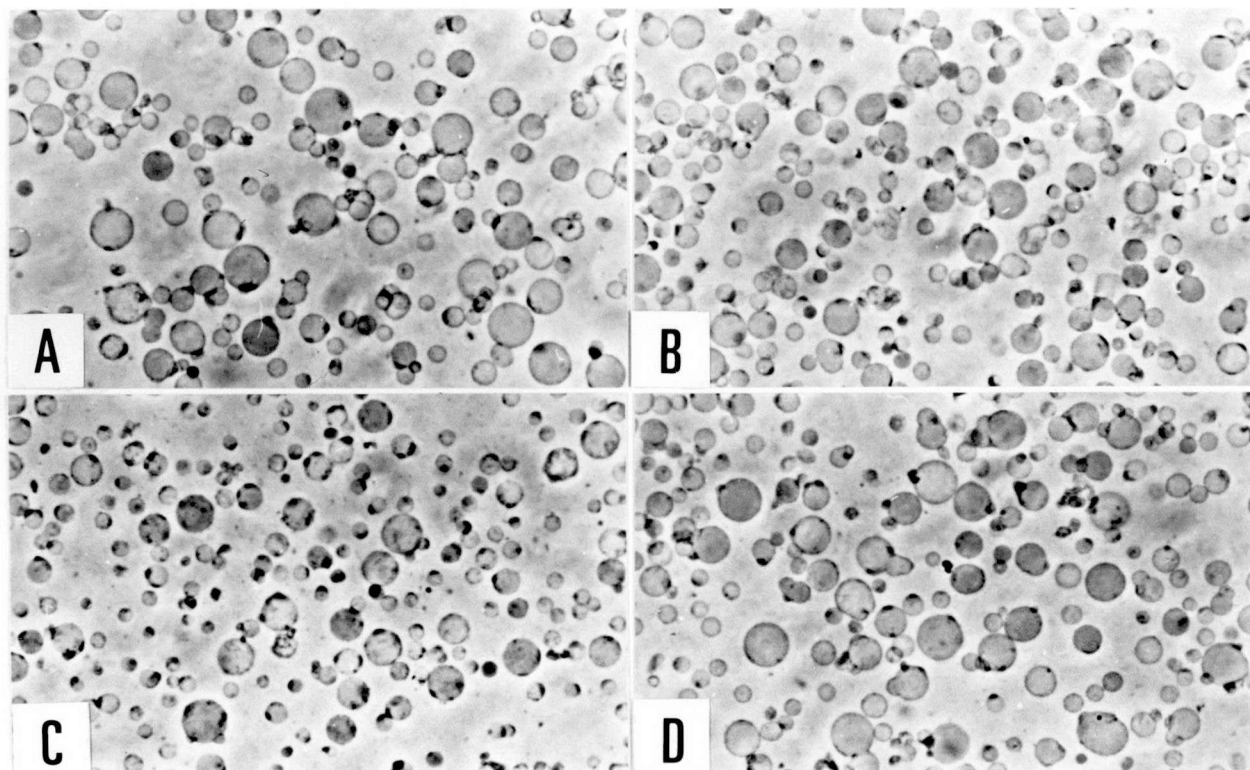


Fig. 10. Effect of calcium ion and MgATP on the fusion reaction of BSA-loaded ghosts. Ghosts were loaded with either calcium ion or MgATP along with BSA(5 %) as described in "Materials and Methods" except that resealed ghosts were washed with and then suspended in TBS containing either calcium ion or MgATP at the same concentrations as during loading. These ghosts were agglutinated by HVJ (6,000 HAU/ml) for 15 min in an ice bath and subsequently incubated for 60 min at 37°. (A), No addition; (B), 1.5 mM calcium chloride; (C), 10 mM calcium chloride; (D), 1.5 mM MgATP. Phase microscopy, x350.

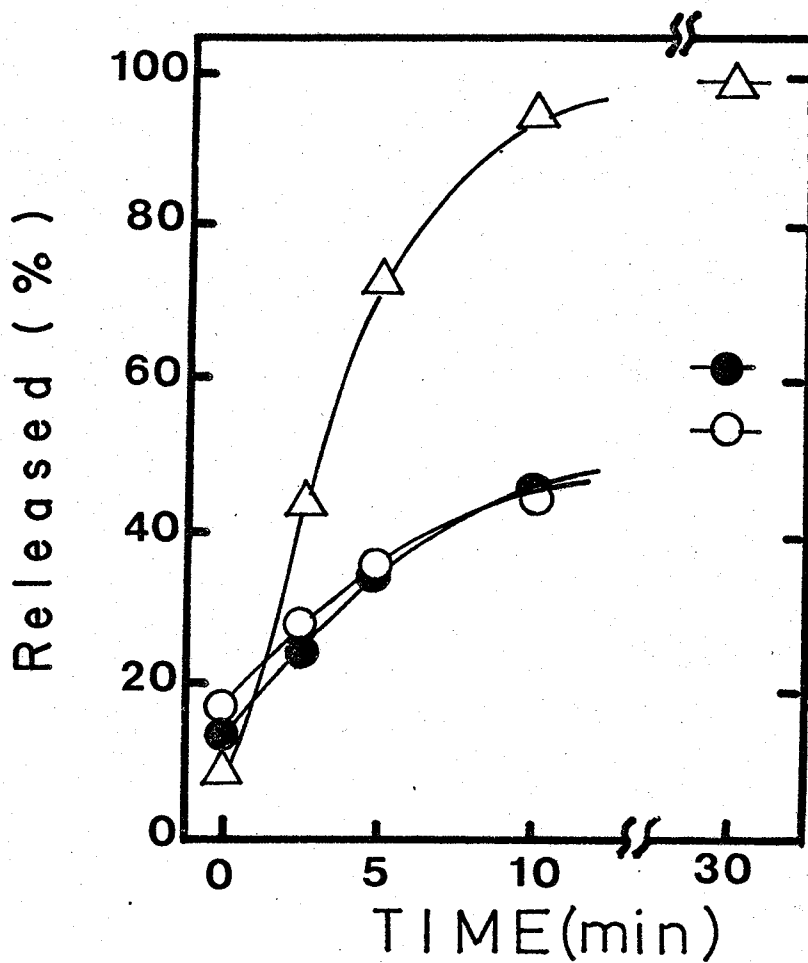


Fig. 11. Virus-induced leakage of internal macromolecules. Either intact erythrocytes (Δ) or ghosts loaded with [^{125}I] BSA (0.5 %, \bullet and 5 %, \circ) were agglutinated by HVJ (3,000 HAU/ml for intact cells and 6,000 HAU/ml for the ghosts) for 15 min in an ice bath then incubated at 37° for different periods. The amount of released macromolecules was determined as described in "Materials and Methods" .

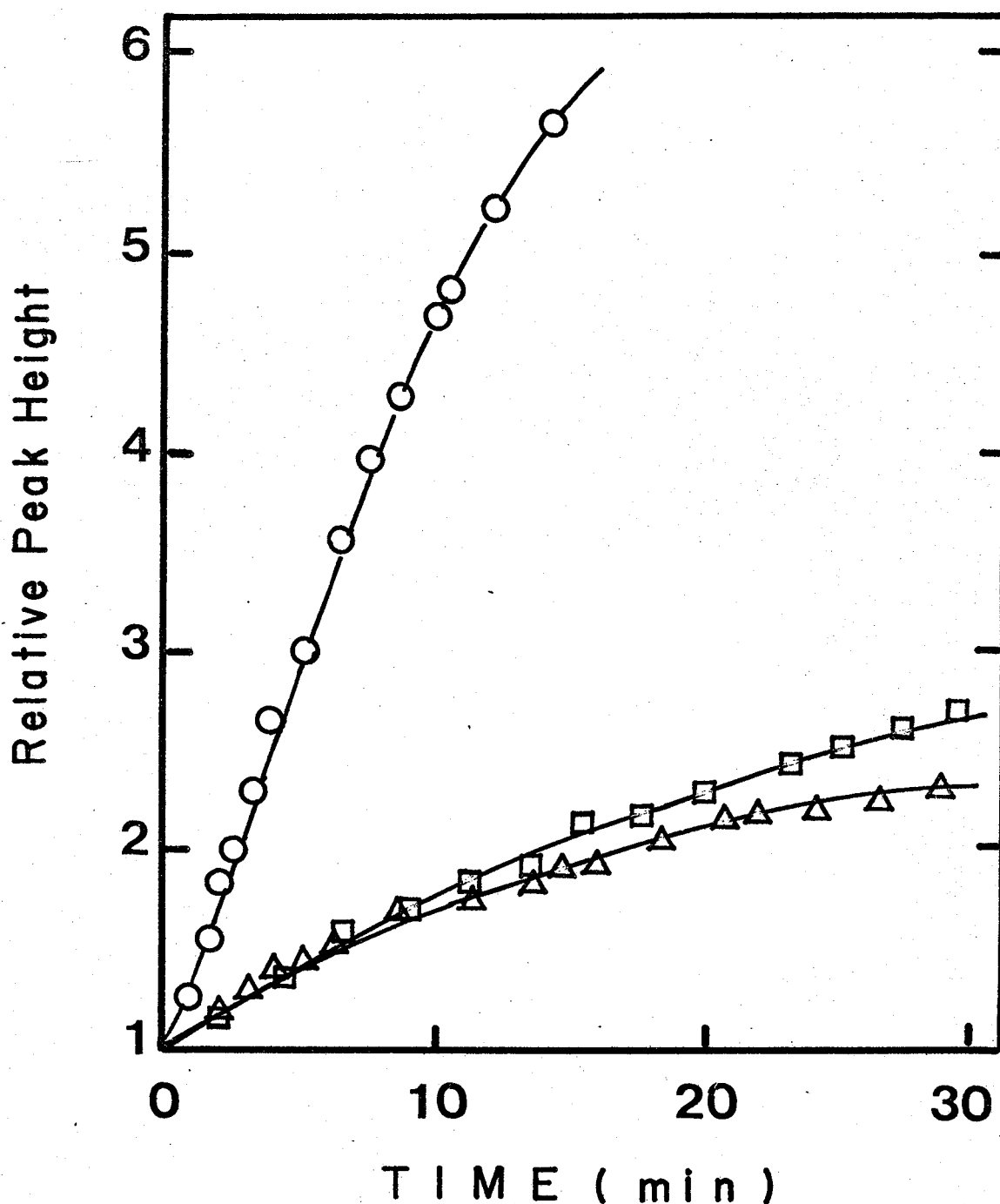


Fig. 12. Phospholipid intermixing between viral envelope and the erythrocyte membrane. Either intact erythrocytes (○) or resealed ghosts prepared in the presence (□) or absence (△) of 5 % BSA were agglutinated by HVJ (a mixture of spin-labeled HVJ (300 HAU/ml) and non-labeled HVJ (5,200 HAU/ml)) for 15 min in an ice bath, then lightly centrifuged. The packed aggregates were taken into a quartz tube and incubated at 37°. Time-dependent changes of ESR spectrum of PC* were followed and relative increase of central peak height was plotted against incubation time at 37°.

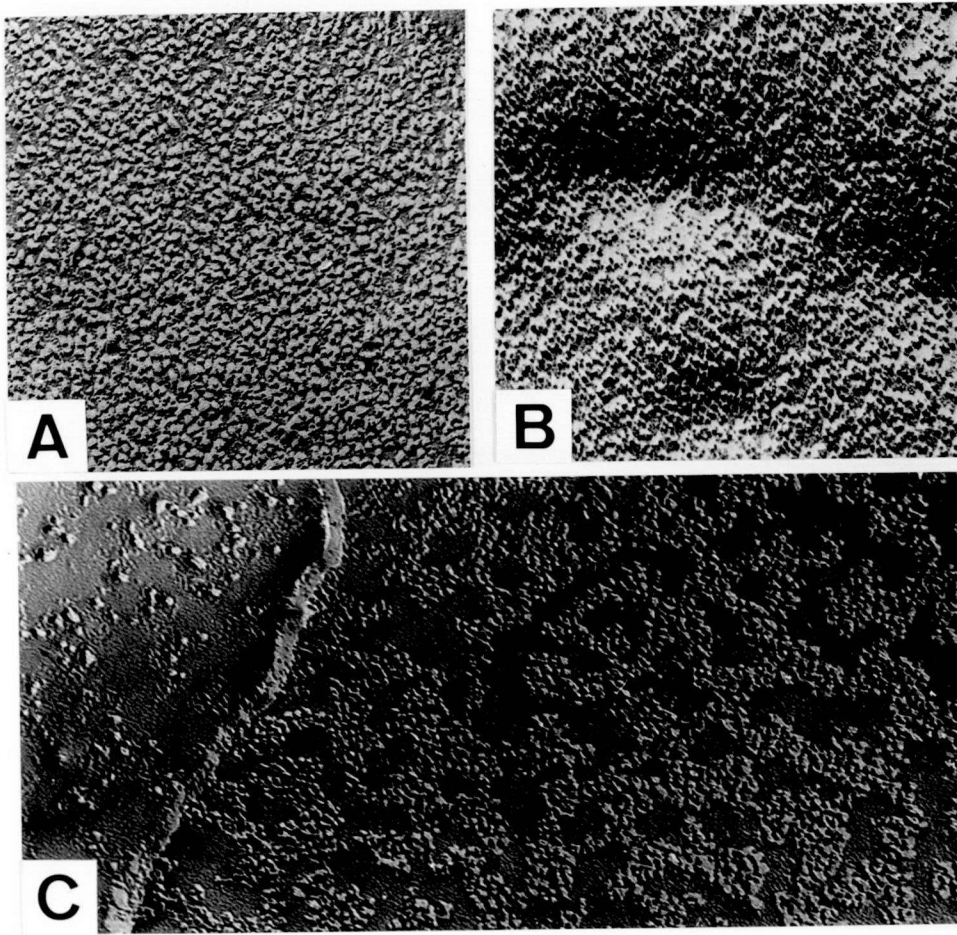


Fig. 13. Rearrangement of intramembrane particles during virus-induced cell fusion reaction. (A), Protoplasmic fracture face (PF) of freeze-cleaved intact erythrocytes. (B), The same as (A) except that intact cells were agglutinated by HVJ for 30 min in an ice bath, then subjected to freeze-fracturing. (C), PF (center to right) and external fracture face (EF, left) of intact cells which were agglutinated by HVJ (800 HAU/ml) for 30 min in an ice bath and incubated at 37° for 30 min. Clustering of intramembrane particles took place all over the fracture face. Note that pits on the EF were also clustered and their pattern seemed to be continued to that of the particles on PF. x 100,000.

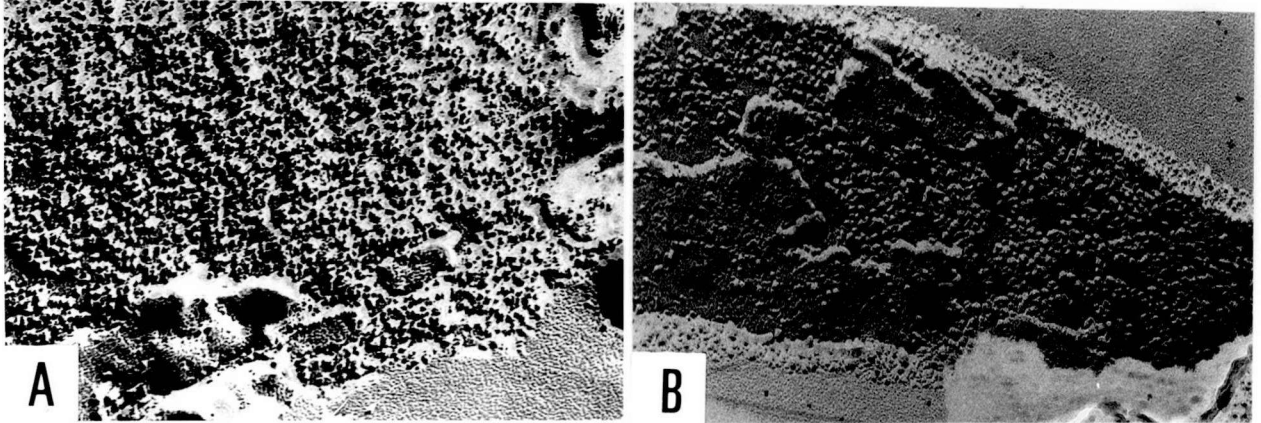


Fig. 14. Rearrangement of intramembrane particles during the fusion reaction of BSA-loaded ghosts. Ghosts which were resealed in the absence (A) or presence (B) of 5 % BSA were agglutinated by HVJ (8,000 HAU/ml) for 30 min in an ice bath and then incubated for 30 min at 37°. Freeze-fracturing was performed as described in "Materials and Methods". Rearrangement of intramembrane particles was observed in (B) but to lesser extent in (A). x 80,000.

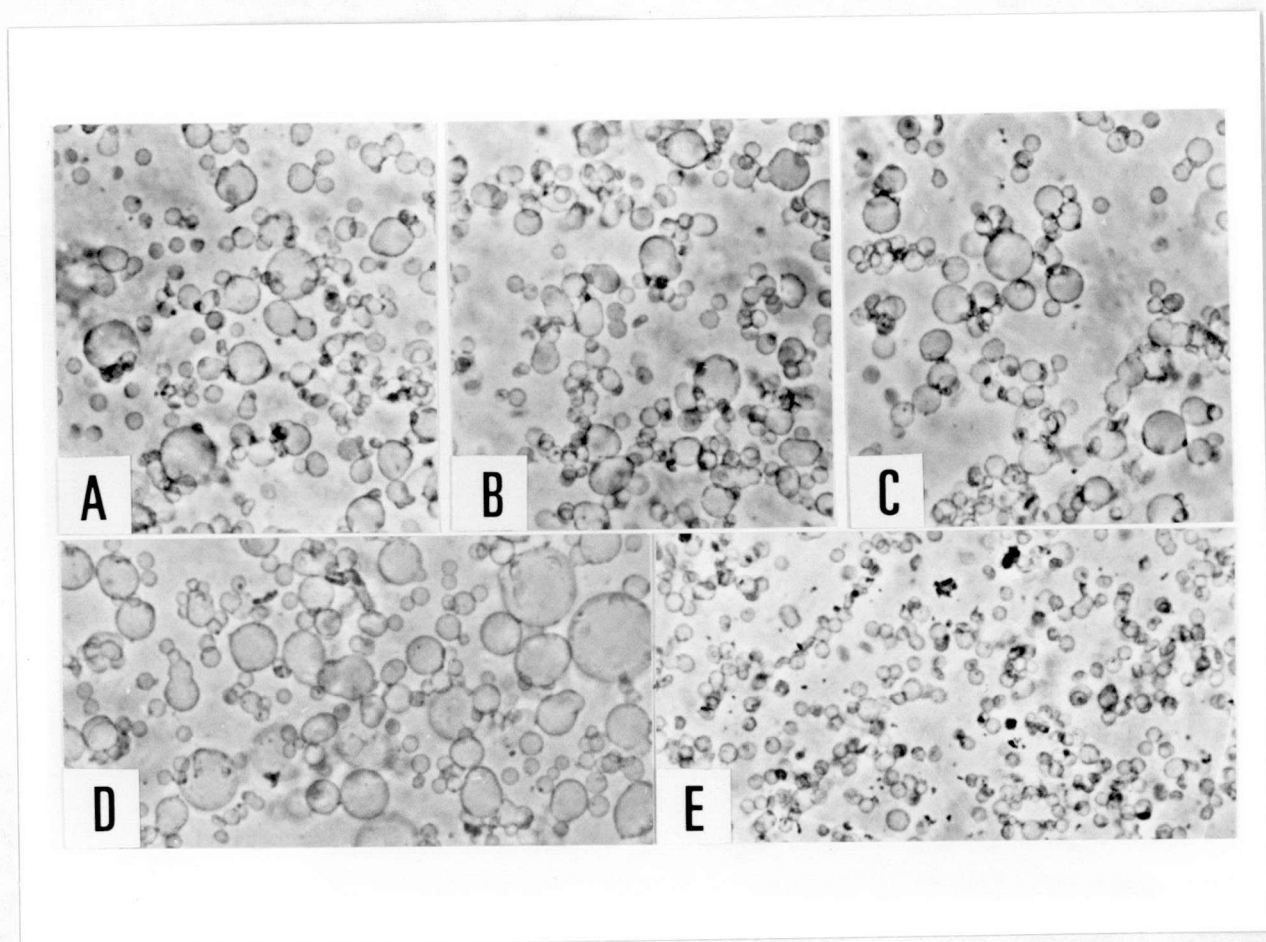


Fig. 15. Restoration of fusion ability of erythrocyte ghosts by loading other macromolecules within ghosts. Ghosts loaded with the following macromolecules were prepared as described in "Materials and Methods". (A), 5 % BSA; (B), 6 % fatty acid-free BSA; (C), 5 % ovalbumin; (D), 30 % dextran T 70; (E), 5 % hemoglobin. These ghosts were agglutinated by HVJ (7,000 HAU/ml for (A,B,E), 3,850 HAU/ml for (C), 4,000 HAU/ml for (D)) for 15 min in an ice bath and then incubated at 37° for 30 min. Phase microscopy, x 350.

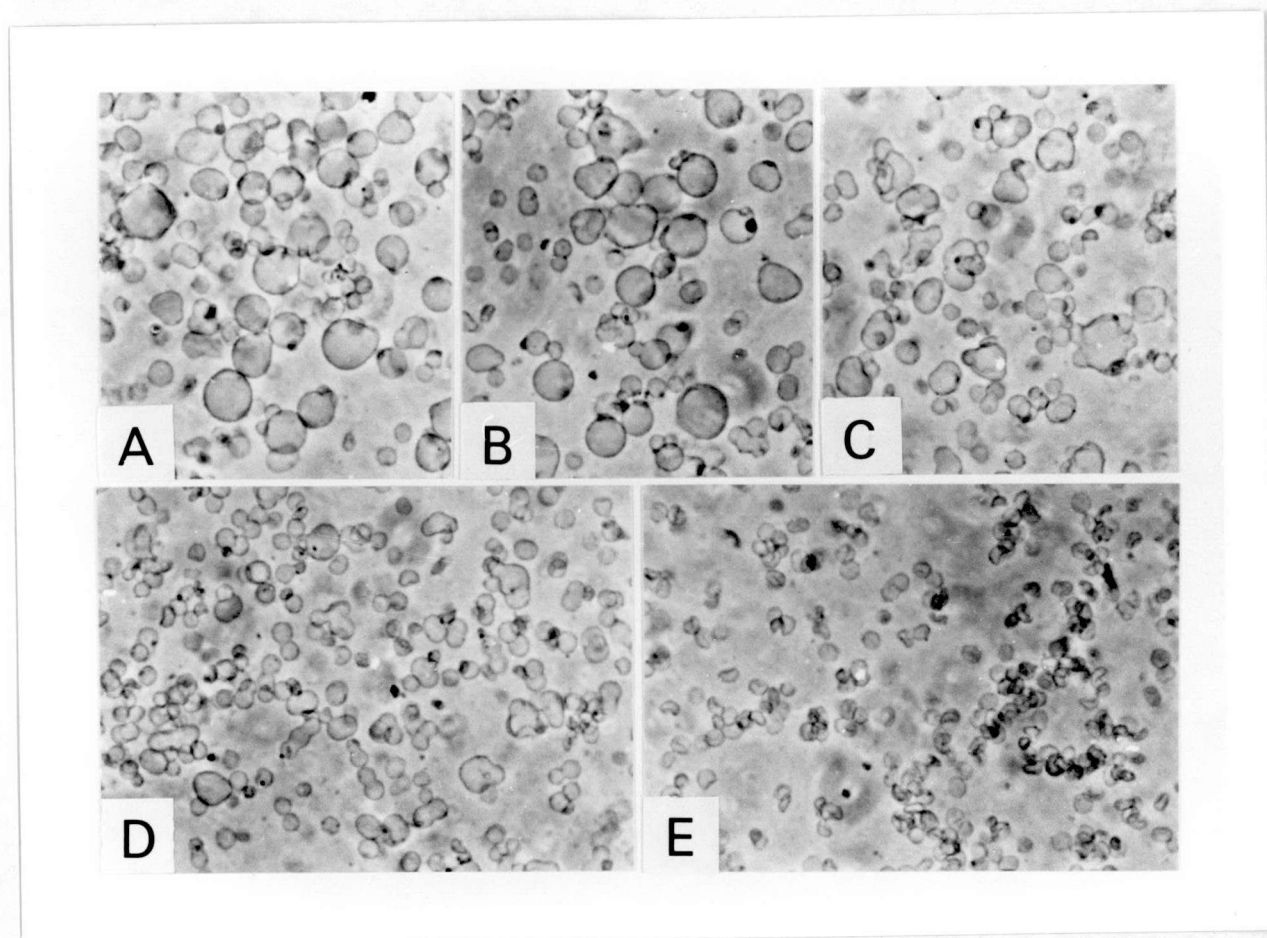


Fig. 16. Effect of externally added BSA on the fusion reaction of BSA-loaded ghosts. BSA-loaded ghosts were agglutinated by HVJ (3,000 HAU/ml) for 15 min in an ice bath, then added BSA of different concentrations and immediately incubated at 37° for 60 min. (A), 0 %; (B), 0.1 %; (C), 0.5 %; (D), 1.0 %; (E), 2.0 %. Phase microscopy, x350.

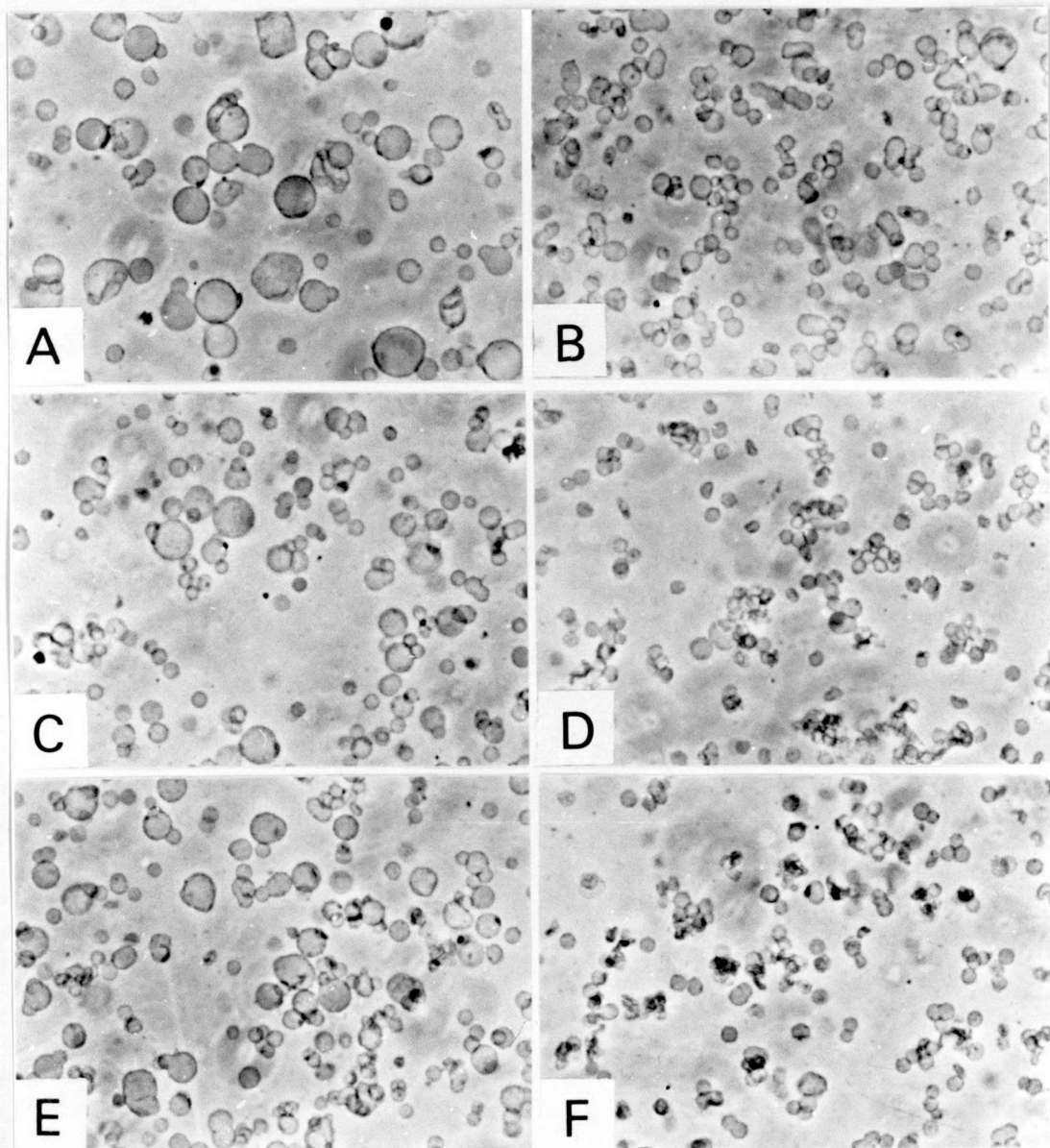


Fig. 17. Effect of externally added macromolecules on the fusion reaction of BSA-loaded ghosts. BSA-loaded ghosts were agglutinated by HVJ (3,000 HAU/ml) for 15 min in an ice bath, then added the following macromolecules of different concentrations and immediately incubated at 37° for 60 min. (A), No addition; (B), 1 % BSA; (C), 0.5 % dextran T 70; (D), 1 % dextran T 70; (E), 0.5 % PVP-40; (F), 1 % PVP-40. phase microscopy, x350.

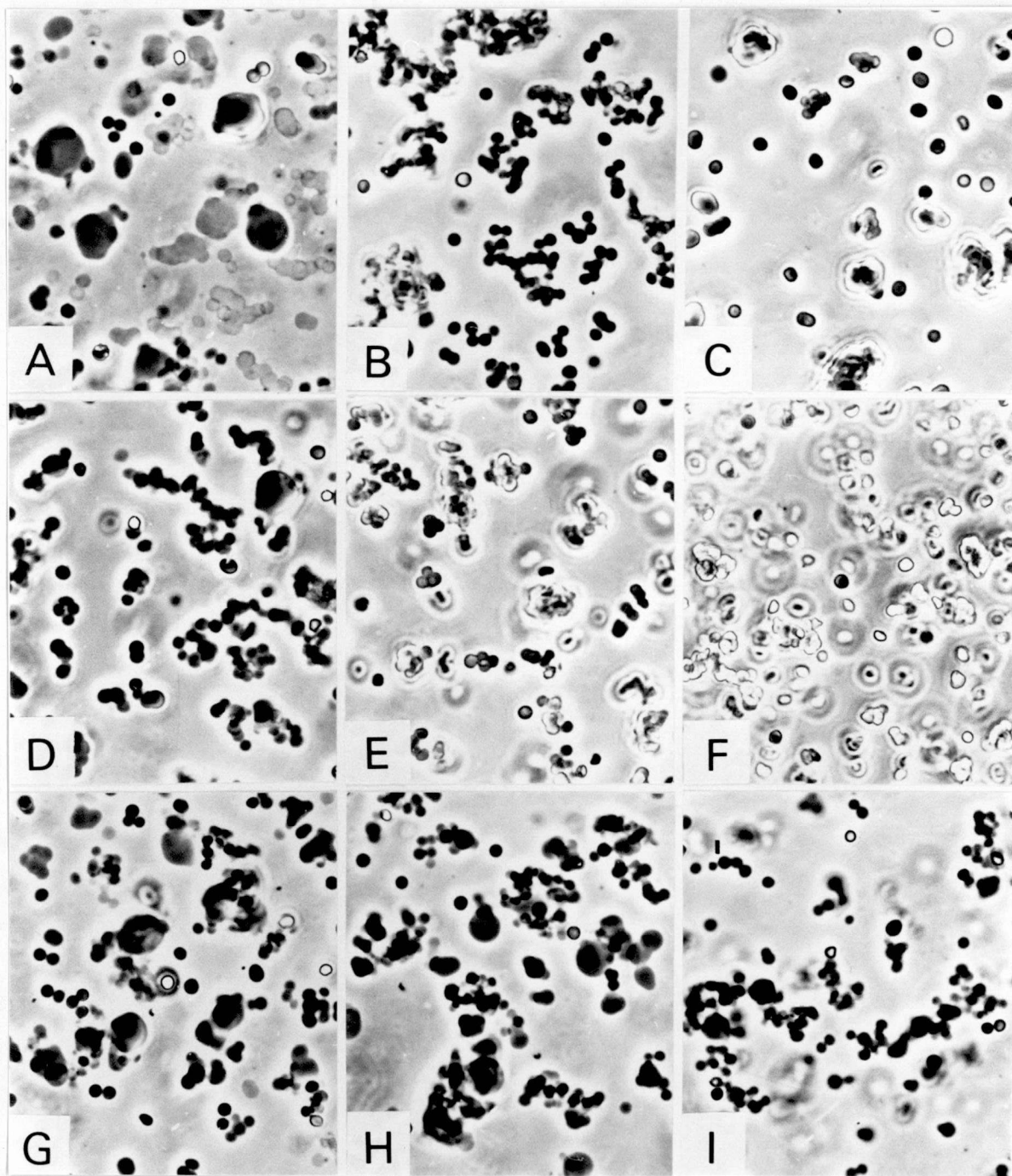


Fig. 18. Effect of externally added macromolecules on the fusion reaction of intact erythrocytes. Intact cells were agglutinated by HVJ (1,500 HAU/ml) for 15 min in an ice bath, then added various concentrations of macromolecules and immediately incubated at 37° for 30 min. (A), No addition; (B), 5 % PVP-40; (C), 10 % PVP-40; (D), 5 % dextran T 70; (E), 10 % dextran T 70; (F), 20 % dextran T 70; (G), 5 % BSA; (H), 10 % BSA; (I), 20 % BSA. Phase microscopy, x350.

II. ROLE OF SPECTRIN IN VIRUS-INDUCED FUSION OF HUMAN ERYTHROCYTE GHOSTS

SUMMARY

A rabbit antibody to human spectrin purified by affinity chromatography was used to study the role of spectrin in HVJ-induced fusion of erythrocytes. This was made possible by the finding reported in the preceding paper that the fusion activity of erythrocyte ghosts can be restored by loading bovine serum albumin (BSA). It was thus found that inclusion of the antibody in BSA-loaded ghosts strongly inhibited the fusion of these ghosts, whereas control globulin caused no effect on the fusion reaction. Fab fragments of the antibody was also ineffective. Maximal inhibition was observed when about 11 moles of the antibody was bound per mole of spectrin, a ratio which also caused maximal precipitation of the antigen-antibody complex. It was concluded that the cross-linking of spectrin by the divalent antibody, but not the binding of the antibody per se, was responsible for the inhibition of fusion reaction. The antibody seemed to affect the final cell-cell fusion step of the whole process, because it did not inhibit the virus-induced cell agglutination and the subsequent fusion of the viral envelope with the cell membrane. Freeze-fracture electron microscopy further showed that inclusion of the antibody, but not control globulin, in BSA-loaded ghosts also prevented the redistribution of intramembrane particles, a phenomenon which was observable during the virus-induced fusion of these ghosts. It was, therefore, likely that the antibody, by forming extensive cross-linkings in the spectrin meshwork, inhibited the molility of intramembrane particles caused by the virus and thus prevented the fusion reaction. It was also found that muscle G-actin included in BSA-loaded ghosts was also considerably inhibitory, although an actin-DNAase I complex and DNAase I alone

did not affect the fusion reaction. This suggested that the G-actin, after conversion to F-actin within the ghosts, combined with spectrin and thus stabilized the spectrin meshwork against the viral action. On the basis of these observations, the mechanism of HVJ-induced fusion of erythrocytes is discussed.

INTRODUCTION

Spectrin is a peripheral protein which is associated with the cytoplasmic surface of the mammalian erythrocyte membrane and comprises about 30 % of the total membrane protein (1-6). It appears to be a heterodimer composed of two polypeptide chains having molecular weights of about 240,000 and 220,000 (5,7). On sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the membrane, these two polypeptides are detected as Band I and II, respectively (1,4). Dialysis of erythrocyte ghosts against buffers of low ionic strength causes the release of spectrin from the membrane together with erythrocyte actin (Band V) and other smaller proteins (3-5,8). Recent studies have suggested that spectrin is an important factor determining the physical properties of erythrocytes, notably the shape and viscoelastic properties of the membrane (10-13). Substantial removal of spectrin from erythrocyte ghosts has been shown to make them spherical and fragile leading to eventual vesiculation into small fragments (4,14,15).

Another postulated function of spectrin is to regulate the lateral mobility of erythrocyte membrane glycoproteins (15-18). Human erythrocyte membrane contains two major glycoproteins, both of which span the membrane matrix and therefore are typical integral membrane proteins (9, 19-21). One of them, called glycophorin (22) or PAS-I (4), is one of the most well characterized membrane proteins and its amino acid sequence has been determined (23). Its carbohydrate content is about 60 % by weight and it carries more than 70 % of the total membrane sialic acid as well as MN antigens, phytohemagglutinin and influenza virus receptors (2,22,23). The other glycoprotein, referred to as Band III (4) or Component a (24), has a molecular weight of about 90,000 and comprises about 25 % of the

membrane protein (1,4). It contains only 5-8 % carbohydrates and is believed to be involved in anion transport (2,25,26). The intramembrane particles observable on the fracture surface of freeze-cleaved erythrocyte membrane are considered to be due to these two glycoproteins (1,27,28). Lateral movement of the glycoproteins (intramembrane particles) in the erythrocyte membrane is extremely restricted compared with those in other cell membrane (15,16,29-31). Antibodies and lectins specific to surface antigens and extruded sugar chains of the glycoproteins are unable to induce the formation of patches and caps of their receptors in erythrocytes (29,30). Furthermore, bleaching of the fluorescence of one half of a single ghost in which Band III glycoprotein has been labeled with fluorescein isothiocyanate is not followed by the recovery of fluorescence, suggesting that Band III protein cannot essentially diffuse in the plane of the membrane (31,32).

Such immobilization of glycoproteins (and intramembrane particles) in the erythrocyte membrane has been suggested to be due mainly to the interaction of the hydrophobic segment(s) of the glycoproteins extruded to the cytoplasmic surface with a cytoskeletal meshwork consisting of spectrin and probably also erythrocyte actin (1,10,15,16,33). Although little aggregation of the intramembrane particles can be induced in the membrane of freshly prepared ghosts by lowering the pH to 5.5, this is made possible after substantial removal of spectrin (15). The particle aggregation takes place optimally at pH 4.0-5.5 (34), which is almost the same with the isoelectric point of spectrin (7,18). It is, therefore, likely that the particle aggregation in the membrane of spectrin-depleted ghosts is due to the isoelectric precipitation of residual spectrin (18). Another support to this view is the finding that anti-spectrin

antibody sequestered into ghosts by osmotic shock causes the transmembrane aggregation of the surface anionic sites due to the sugar chains of glycophorin, although the monovalent Fab fragment of the antibody is ineffective (17). Furthermore, cross-linking of membrane proteins with bifunctional imidoesters has shown that a part of Band III protein is situated very close to spectrin in the membrane (35). All these observations are consistent with the view that there are direct interactions between the membrane glycoprotein(s) and the spectrin meshwork.

It has been reported that similar redistribution of the intramembrane particles can be seen during the virus-induced fusion of erythrocytes and their ghosts (36-40), those induced by non-viral fusogenic agents, as well as other membrane fusion processes (41-45). A proposal has, therefore, been made that the fusion of closely apposed membranes takes place in the region where the intramembrane particles are excluded and the smooth lipid surface is exposed (46,47). If this is actually so, then modifications of the spectrin meshwork by anti-spectrin antibody or other agents acting specifically on spectrin should affect the mobility of the particles resulting in alterations of the fusion efficiency of erythrocytes and their ghosts.

BSA-loaded erythrocyte ghosts developed in the preceding paper seems to provide a suitable experimental system to examine this hypothesis, because this system permits studies of the effects of various reagents sealed into the ghost on the fusion activity. In this paper, therefore, we examined the effects of anti-spectrin antibody and some other agents included in BSA-loaded ghosts on the fusion efficiency of the ghosts. The results to be reported in this

paper support strongly the hypothesis described above that the rearrangement of the intramembrane particles is actually a prerequisite to the virus-induced fusion of the ghosts.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA, fraction V), pancreatic DNAase I (DN-EP), Tricine (N-tris[hydroxymethyl]methyl glycine) and phenyl-methylsulfonyl fluoride were obtained from Sigma Chemical Co., dextran T 70, Sephadex G-25 and G-75, Sepharose 4B were from Pharmacia Fine Chemicals, glutaraldehyde was from Ladd Research Industries Inc. and Na^{125}I (carrier free) was from New England Nuclear Corp. Sodium deoxycholate (DOC) was obtained from Difco Laboratories and used without further purification. 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 (48). The other chemicals used were of reagent grade or of the highest grade available. The buffer used for cell fusion experiments was TBS, the composition of which was described in the preceding paper.

Cells and Virus. Human blood, stored in ACD solution, from donors of different blood types, was obtained from a blood bank (Midori-juji Co, Osaka) and used within four weeks after drawing. For the fusion experiment of intact erythrocytes, cells were washed four times with PBS (phosphate-buffered saline, consisted of 0.9 % NaCl and 5 mM sodium phosphate, pH 7.6), twice with TBS and finally suspended in TBS to give a concentration of about 10 % (v/v). HVJ (Sendai virus), Z strain, was propagated in embryonated eggs. The infected chorioallantoic fluid was purified by two cycles of differential centrifugation (49) and suspended in TBS. The dose of the virus was expressed in terms of its hemagglutination unit (HAU) which was determined by Salk's pattern method (50).

Purification of Spectrin. Spectrin was extracted from erythro-

cyte ghosts by dialysing against 1 mM EDTA (pH 10.3) at 4° for 18-20 h. Extracted spectrin was concentrated by vacuum dialysis and then applied to a Sepharose 4B column (3.8x90 cm) and eluted with 10 mM DOC, 10 mM Tris·HCl, 0.1 mM EDTA, pH 9.0 (51). Spectrin thus purified was further passed through Sephadex G-25 to remove DOC.

Preparation and Purification of Anti-spectrin Antibody. Antibody against purified spectrin was raised in male rabbits with Freund's complete adjuvant. The antisera were first fractionated by ammonium sulfate precipitation, then further purified by affinity chromatography. Spectrin was partially purified by isoelectric precipitation where pH of the EDTA extract was adjusted to 4.8 with 20 mM sodium acetate and then coupled to CNBr-activated Sepharose 4B which was prepared by the method of Cuatrecasas (52). Partially fractionated antibody was applied on the spectrin-Sepharose column (2-3 mg spectrin/ml of packed gel) at room temperature and washed with 20 mM sodium borate, 0.15 M NaCl, pH 8.0 until the absorbance of the effluent was less than 0.05. Elution was performed at 4° with 0.17 M glycine·HCl pH 2.8. Fractions whose absorbance at 280 nm is >0.05 were pooled and immediately titrated to pH 7.0 with 0.5 M sodium bicarbonate, pH 9.0. Neutralized antibody was dialysed against TBS and after concentration by vacuum dialysis stored at -80°. Fab fragments were prepared by the method of Porter (53) except that before applying to carboxymethyl cellulose column papain digests were subjected to Sephadex G-75 to remove papain. Control immunoglobulin was prepared from non-immunized sera by ammonium sulfate precipitation and subsequent DEAE cellulose column chromatography (54).

Preparation of Muscle Actin. Muscle actin was prepared from an acetone powder of rabbit skeletal muscle according to the procedure of Spudich and Watt (55).

Pretreatment of DNAase I with Phenylmethylsulfonyl Fluoride.

DNAase I (DN-EP, electrophoretically purified) was once reacted with 0.05 % phenylmethylsulfonyl fluoride for 20 min at room temperature and then extensively dialysed against 2 mM Tris HCl-0.2 mM ATP, pH 8.0.

Preparation of Ghosts and Loading of BSA, Anti-spectrin Antibody and Other Proteins.

Erythrocyte ghosts were prepared according to Dodge et al. (56). Washed erythrocytes were hemolysed by adding 20 vol of hemolysing buffer (5 mM sodium phosphate, pH 8.0) and immediately centrifuged at 15,000 rpm for 20 min with Sorvall SS-34 rotor at 4°. The lysed cells were washed once with the same buffer. After centrifugation, one vol of packed ghosts was mixed with four vol of TBS containing BSA and other proteins (loading medium) and allowed to stand for 15 min at 0°, then resealed by incubating at 37° for 60 min. When actin or DNAase I was loaded along with BSA, one vol of packed ghosts were first mixed with one vol of 25 % BSA solution and two vol of actin or DNAase I (dissolved in 2 mM Tris·HCl-0.2 mM ATP, pH 8.0) and preincubated on ice bath for 10 min then another one vol of concentrated buffer (5-fold conc. TBS) was added to restore isotonicity and further incubated on ice for 10 min, then at 37° for 60 min to allow re-sealing. Ghosts loaded with BSA and other proteins were washed twice with TBS and suspended in TBS.

Fusion Experiments. Unless otherwise stated, 0.19 ml of 2 % ghosts was mixed with 0.01 ml of HVJ and kept in an ice bath for

15 min to allow agglutination. The resulting aggregates were incubated at 37° for 30-60 min and then chilled in ice to terminate the fusion reaction. When the effect of externally added antibody was examined, 0.02 ml of 10 % ghosts or intact erythrocytes were mixed with 0.005 ml of HVJ and agglutinated in an ice bath for 15 min. The antibody (0.01 ml) was then added and incubated at 30° for 30 min. Additional incubation was performed for 30 min at 37° for intact cells but at 30° for the ghosts. Fused cells or ghosts were examined by phase contrast microscopy.

Iodination of BSA and Anti-spectrin Antibody. Radioiodination of BSA or the antibody was performed by a chloramine T method (57) as described in the preceding paper. Iodination was started by the addition of chloramine T (20 µg/ml) and continued at 4° for 10 min with gentle shaking. The reaction was terminated by the addition of sodium metabisulfite (20 µg/ml). Iodinated protein was passed through Sephadex G-25 to remove low molecular compounds. After concentration by vacuum dialysis, iodinated protein was stored at -20°.

Measurement of the Amount of Anti-spectrin Antibody Bound to the Erythrocyte Membrane. Ghosts loaded with BSA (5 %) and ¹²⁵I-labelled anti-spectrin antibody (4.6 mg/ml), which were prepared as descibed above, were lysed in 12 vol of hemolysing buffer and then centrifuged at 16,000 rpm for 30 min. The packed ghosts were once washed with the same buffer and then the amount of bound antibody was determined in a well-type scintillation spectrometer (Aloka Auto Well Gamma System, JDC-751).

Measurement of Virus-Induced Lysis. Ghosts (0.19 ml) loaded with [¹²⁵I]BSA and other protein were agglutinated by HVJ (0.01 ml) in an ice bath for 15 min then incubated at 37° for different

periods of time. The fusion reaction was terminated by the addition of cold TBS (2.80 ml) and then immediately centrifuged at 3,000 rpm for 15 min. The amount of released [125 I]BSA in the supernatant was determined in a well-type scintillation spectrometer.

Preparation of Spin-Labeled HVJ. Spin-labeled HVJ was prepared according to the procedure of Maeda et al. (58). PC* (2 mg/ml) suspended in TBS was sonicated under a stream of nitrogen gas in an ice bath and then centrifuged at 100,000xg for 60 min at 4°. The supernatant was mixed with equal vol of HVJ (the molar ratio of the viral lipids to PC* was about 0.2) and incubated at 37° for 6 h. Spin-labeled HVJ (HVJ*) was first washed with TBS which contained BSA (10 mg/ml) then with TBS and finally suspended in TBS. The resulting HVJ was densely labeled with PC* and the electron spin resonance (ESR) spectrum of HVJ* was broadened by spin-spin exchange interaction.

Assay of Phospholipid Intermixing between Viral Envelope and the Erythrocyte Membrane. Ghosts were agglutinated by a mixture of HVJ* (300 HAU/ml) and non-labeled HVJ (5,200 HAU/ml) for 15 min in an ice bath, then pelleted by light centrifugation at 2,000 rpm for 5 min. The packed agglutinated ghosts were immediately taken into a quartz capillary tube and ESR spectrum was recorded at 37° on a JEOLCO ME-X spectrometer. Increase in the central peak height was measured and plotted against incubation time.

Freeze-Fracture Electron Microscopy. The samples were fixed by the addition of an equal vol of 4 % glutaraldehyde dissolved in TBS and prewarmed to the temperature of the reaction. Fixation was performed for 40 min at 37° or 2 h at 4° in case of control cell aggregates which was not incubated at 37°. After three washes with TBS, the fixed samples were suspended in TBS containing 20 %

glycerol and allowed to stand at 4° overnight. Freeze-fracturing was performed at standard conditions by a Hitachi freeze-fracture apparatus. Electron micrographs were taken by a Hitachi electron microscope.

Measurement of the Number of Ghosts. The number of ghosts was counted with a Coulter Counter Model A (Coulter Electronics Ltd.) fitted with a 100 μ m aperture. The numbers obtained were reproducible within ± 2 % of the mean value.

Assay. Protein was determined by the method of Lowry et al. (45).

RESULTS

Characterization of Anti-spectrin Antibody. To examine the role of spectrin in the virus-induced fusion of erythrocyte ghosts, it is critical that (1) the antibody is monospecific for spectrin and does not bind to or precipitate other membrane components and (2) the content of spectrin-specific antibody is relatively high because effective concentrations of spectrin-specific antibody to cause significant changes of the fusion reaction are more than 1 mg protein per ml. To satisfy these criteria, crude anti-serum, which was raised in a rabbit with purified spectrin as an antigen, was further purified by affinity chromatography. Spectrin, partially purified by isoelectric precipitation (7) (Fig. 1), was coupled with BrCN-activated Sepharose 4B and used for purification of anti-spectrin antibody. Monospecificity of purified anti-spectrin obtained was confirmed by double diffusion test. As shown in Fig. 2, the purified antibody formed only one precipitin band both with whole erythrocyte membrane proteins solubilized with sodium deoxycholate (10 mM) and with purified spectrin and each single precipitin band fused with each other.

Fig. 3 shows the antigen-antibody precipitation curve. About 63 % of the antibody could be precipitated with increasing the amount of added spectrin up to 30 μ g. Further addition of spectrin, however, resulted in the decrease of precipitation of the antibody (data not shown). The molar ratio of the antibody to spectrin at the condition for maximal precipitation was approximately 15-20 when assuming the molecular weight of spectrin was 460,000. The content of the specific antibody which was precipitable with spectrin was slightly lower than that obtained in other studies (12,17),

but it might be due to the partial denaturation of the antibody during the elution of bound antibody from an affinity column with acidic buffer and also subsequent iodination of the antibody. Fig. 3 also shows that preincubation of spectrin with Fab fragments prepared from the antibody resulted in extensive inhibition of the subsequent precipitation of the antibody and an amount of Fab fragments, that was 1.4 times the weight of bivalent antibody, completely inhibited the precipitation of the antibody, confirming the binding activity of Fab fragments to spectrin.

Effect of Anti-spectrin Antibody on the Fusion of Erythrocyte Ghosts.

To elucidate the role of spectrin in virus-induced fusion of human erythrocyte ghosts, anti-spectrin antibody was incorporated into ghosts along with BSA and the effect of the included antibody on the fusion reaction was examined. As shown in Fig. 4, included antibody was inhibitory for the fusion reaction and it almost completely inhibited the fusion at the concentration of 4 mg/ml (Fig. 4B and C), whereas inclusion of control immunoglobulin at the same concentration did not affect the fusion activity of the ghosts (Fig. 4). Furthermore, monovalent Fab fragments, which were loaded within ghosts along with BSA at 4 mg/ml, did not affect the fusion reaction either (Fig. 4D), indicating that it was the cross-linking of spectrin by bivalent antibody, rather than the binding per se, that caused the extensive inhibition of the fusion reaction. The amount of the antibody bound to the erythrocyte membrane at the condition for maximal inhibition of the fusion reaction was measured using ^{125}I -labeled antibody and it was found that the molar ratio of the bound antibody to spectrin was about 1:1, when assuming that spectrin comprises about 30 % of

the total membrane proteins. This value was in good agreement with that obtained for the maximal precipitation of the antigen-antibody complex, again confirming that the antibody exerted its inhibitory effect by cross-linking the spectrin meshwork.

It is critical for the restoration of the fusion activity of erythrocyte ghosts that ghosts are resealed and retain internal BSA. To examine the effect of the included antibody on the resealing process of ghosts, ghosts loaded with the antibody along with BSA were analyzed by dextran T 70 discontinuous density gradient centrifugation. As shown in Fig. 5, most of the antibody-treated ghosts remained on the top of the rotor tube after density equilibrium was attained and the recovery of acetylcholine esterase activity (a typical marker enzyme of external surface) at the buoyant fraction was more than 90 % of the total activity. These results indicate that most of the antibody-treated ghosts were sealed and therefore the inhibition of the fusion reaction by the antibody could not be attributable to the failure in resealing.

Since virus-induced cell fusion involves at least three different steps, *i.e.*, agglutination of cells, fusion between viral envelope and cell membrane ("envelope fusion") and fusion between adjacent cell membranes ("cell-cell fusion"), effect of the antibody on the two preceding steps was next examined. As shown in Fig. 6, the included antibody did not affect the agglutination of the ghosts, since there was no significant difference in the degree of virus-induced agglutination between ghosts into which either control immunoglobulin or anti-spectrin antibody were incorporated.

In addition, it seems that included antibody did not affect the envelope fusion either. Since virus-induced lysis of ghosts

can be taken as an index of envelope fusion (60-62), the effect of the antibody on the virus-dependent leakage of included BSA was examined. As shown in Fig. 7, the leakage of included BSA from ghosts was not affected by the antibody and more than 80 % of internal BSA leaked out of ghosts irrespective of the presence or absence of the antibody.

These observation was further supported by another possible measurement of envelope fusion which makes use of spin-label technique. This method utilizes marked decrease in the exchange broadening of ESR spectrum which takes place when densely spin-labeled membranes are mixed with unlabeled membranes (58,63). The degree of the disappearance of exchange broadening can be expressed as an increase in the central peak height. As shown is Fig. 8, when densely labeled HVJ was mixed with ghosts and then incubated at 37°, central peak height of ESR spectrum rapidly increased irrespective of the presence or absence of the antibody within ghosts, confirming that the antibody did not affect the envelope fusion.

Effect of Anti-spectrin Antibody on Virus-Induced Rearrangement of Intramembrane Particles. Since the antibody did not affect the envelope fusion, the antibody may directly inhibit the final step in the fusion reaction, i.e., cell-cell fusion. It is considered that rearrangement of intramembrane particles may be required for the completion of cell-cell fusion (36,39,46,47), thus effect of the antibody on the distribution of intramembrane particles was examined. As shown in Fig. 9A and B, the inclusion of the antibody (5 mg/ml) within ghosts along with BSA did not alter the dispersed distribution of the intramembrane particles, suggesting that extensive cross-linking of spectrin by the antibody by itself

did not cause particle aggregation under the isotonic salt condition.

Upon the agglutination of ghosts by HVJ at 0° and subsequent incubation at 37°, rearrangement of the particles took place and smooth areas devoid of the particles were locally formed in the case of ghosts within which control immunoglobulin was included, corresponding to the extensive fusion of the ghosts (Fig. 9C). Inclusion of the antibody within ghosts, however, inhibited the rearrangement of intramembrane particles and after 30 min incubation at 37° the particles were still randomly distributed (Fig. 9D), where fusion reaction was also almost completely inhibited. Close correlation between the inhibition of both the fusion reaction and rearrangement of intramembrane particles strongly indicates that rearrangement of the particles, which may result in the exposure of bare phospholipid bilayer, is a prerequisite for the completion of cell-cell fusion. It was noted that rather large fracture faces could be easily obtained when the antibody-treated ghosts were freeze-fractured after the incubation with HVJ at 37° for 30 min, whereas such large fracture faces could be scarcely obtained when fused ghosts were subjected to freeze-fracturing, suggesting that the ease in getting large fracture faces may be correlated with the inhibition of the fusion reaction.

Inhibition of the Fusion Reaction by Externally Added Antibody.

Envelope fusion, which is considered to precede the cell-cell fusion, results in the partial lysis of cells, during which externally added antibody can be incorporated into cells. Assuming that there may be a lag time between envelope fusion and cell-cell fusion and further that the incorporated antibody rapidly cross-links spectrin meshwork before the cell-cell fusion takes place,

it may be expected that the externally added antibody inhibit the fusion reaction under appropriate conditions. To examine the effect of the external antibody on the fusion reaction of erythrocyte ghosts, the reaction temperature was lowered to 30° to slow down the overall reaction and prolong the lag time between envelope and cell-cell fusion. As expected, the externally added antibody (4.3 mg/ml) significantly, but not completely, inhibited the fusion reaction of ghosts (Fig. 10C) whereas the addition of control immunoglobulin at the same concentration did not affect the fusion reaction (Fig. 10B), confirming that the inhibitory effect of the antibody was not due to the common effect of external macromolecules to inhibit the fusion reaction. Furthermore, the inhibitory effect of the externally added antibody could not be attributed to any contamination which could bind to the extracellular surface of the ghosts, because the fusion activity of the ghosts, which was pre-incubated with the antibody at 37° for 30 min then washed once with TBS, was not affected by such treatment (Fig. 11).

It was also examined whether externally added antibody could inhibit the fusion of intact erythrocytes. Since cell-cell fusion was more significantly slowed down than hemolysis (*i.e.*, envelope fusion) at 30° compared with 37°, intact cells which were agglutinated by HVJ in the presence or absence of the external antibody were first incubated at 30° for 30 min to introduce the antibody into cells during partial hemolysis, then transferred to 37° and incubated for 30 min. As shown in Fig. 12, the addition of the antibody (13.7 mg/ml) resulted in both the considerable inhibition of the fusion reaction and promotion of hemolysis. Control immunoglobulin which was added at the same concentration was without

effect (data not shown). When the temperature was kept at 37° throughout the fusion reaction, inhibition of the reaction, but to less extent, could be also observed (data not shown).

Effect of Muscle Actin on the Fusion Reaction of Erythrocyte Ghosts.

There is an increasing amount of evidence suggesting that muscle actin specifically interact with spectrin (64-67). Recently Birchmeier and Singer have shown that ATP-induced shape changes of erythrocyte ghosts, in which spectrin is considered to play a critical role, was significantly inhibited by included muscle G-actin, while actin previously complexed with pancreatic DNAase I or DNAase I alone did not affect the shape changes (68). It seemed interesting, therefore, to examine the effect of muscle actin on the fusion reaction of erythrocyte ghosts.

Muscle G-actin, together with BSA, was loaded within ghosts in a low salt medium to prevent the actin polymerization, then isotonicity was restored by the addition of concentrated buffer solution (5-fold conc. TBS) and incubated at 37° to reseal the ghosts. Fig. 13 shows the effect of muscle actin thus included within ghosts on the fusion reaction of erythrocyte ghosts. Included muscle actin inhibited the fusion reaction with increasing the concentration up to 2 mg/ml, where almost complete inhibition could be obtained. When actin was complexed with DNAase I (69) before loading into ghosts, its inhibitory effect was almost reversed (Fig. 14D), whereas DNAase I alone did not inhibit, but rather slightly stimulated, the fusion reaction (Fig. 14C), being in good agreement with the results obtained by Birchmeier and Singer on the ATP-induced shape changes.

DISCUSSION

Rearrangement of Intramembrane Particles in a Prerequisite for Virus-Induced Fusion of Erythrocytes. Recent studies with freeze-fracture electron microscopy have shown that redistribution of intramembrane particles takes place during the fusion of intact erythrocytes and their ghosts induced by virus or non-viral fusogenic agents (36-40). Similar changes in the distribution of the particles are observed in other membrane fusion phenomena (41-45). These close correlation between rearrangement of the particles and membrane fusion, therefore, suggests that redistribution of the particles is an essential step in the cell fusion reaction (46,47). As presented in the preceding paper, fusible ghosts containing BSA showed significant particle aggregation upon incubation with HVJ, whereas non-fusible ghosts lacking internal BSA underwent lesser rearrangement of the particles. The results presented in this paper that anti-spectrin antibody could inhibit both virus-induced particle aggregation and cell-cell fusion, is another, but more convincing evidence indicating that rearrangement of intramembrane particles is a prerequisite for cell-cell fusion. Rearrangement of the particles produces smooth area which is devoid of the particles and fusion between adjacent cell membranes may occur at such smooth region(s) where bare lipid bilayer is exposed, since there is evidence that fusion between simple lipid bilayer (i.e., liposome fusion) can be observed under appropriate conditions (70-74).

How Does HVJ Induce Rearrangement of Intramembrane Particles?

There is some evidence indicating that lateral mobility of membrane intercalated glycoproteins (or intramembrane particles) is extremely limited (15,16,29-31), when compared with other cell membranes (16). It is considered that this immobilization of memb-

rane glycoproteins is mainly due to the specific interaction between cytoplasmic segment(s) of membrane glycoproteins and spectrin meshwork, which associates with cytoplasmic surface of the erythrocyte membrane (1,10,15,16,33). Elgsaeter and Branton showed that the extent of the particle aggregation, which could be observed when ghosts were incubated in hypotonic medium at acidic pH, was dependent on the amount of released spectrin and actin from the ghosts and substantial removal of spectrin is required for maximal particle aggregation (15). Optimal pH for maximal particle aggregation was about 5.0 and it is almost the same as the isoelectric point of spectrin and actin complex (18), suggesting that particle aggregation was induced by local isoelectric precipitation of residual spectrin to which intramembrane particles were anchored. They also showed that particle aggregation could be induced at neutral pH, when ghosts from which spectrin and actin were substantially eluted were incubated in isotonic medium (45). These observations suggest that considerable structural alterations of spectrin meshwork and/or partial release of spectrin and actin from ghosts may occur during the virus-induced fusion reaction, since increase in mobility of intramembrane particles is required for the particle aggregation. It may be unlikely, however, that substantial removal of spectrin and actin complex from the ghosts takes place during the fusion reaction, because any significant release of spectrin and actin could not be observed during the fusion reaction of intact erythrocytes (data not shown). Thus increase in mobility of intramembrane particles during the fusion reaction may be mainly due to the structural alterations or damages of spectrin meshwork induced by viral action. Removal of terminal sialic acid may also stimulate the rearrangement of the particles by reducing charge repulsion.

Swelling of cells after fusion reaction may further suggest that integrity of spectrin meshwork was significantly perturbed and it could no longer maintain the original shape.

Recently, Fowler and Branton have shown that lateral mobility of intramembrane particles, which was measured as diffusion rate of fluorescein isothiocyanate-labeled membrane glycoprotein(s) (practically identical to Band III glycoprotein), was considerably increased after virus-induced cell fusion (32), again indicating that either spectrin meshwork itself or interaction between the particles and the meshwork were modified by viral action.

Anti-spectrin antibody, when included within ghosts, causes extensive cross-linking of spectrin and may reinforce the spectrin meshwork, thus counteracts the viral action to perturb the meshwork. This view may be supported by the fact that (1) included Fab fragments, which could bind to spectrin but could not cross-link it, did not affect the fusion activity of ghosts, (2) the molar ratio of bound antibody to spectrin at the maximal inhibition of the fusion reaction was in good agreement with that for the maximal precipitation of the antigen-antibody complex.

The way HVJ induces structural alterations of spectrin meshwork resulting in the increase in the mobility of intramembrane particles, however, has not yet been clear. Since included antibody did not affect the intermediate steps of cell fusion reaction up to envelope fusion, it may be likely that certain viral component(s) may become to directly interact with spectrin meshwork after envelope fusion in which viral envelope is integrated into the cell membrane, thus perturbing the integrity of spectrin meshwork and/or the interaction between intramembrane particles and spectrin mesh-

work. It seems that identification of the critical component(s) responsible for the structural alteration of spectrin meshwork may be one of the most important problem for the understanding of the mechanism of cell fusion.

Does Anti-spectrin Antibody Alone Induce Rearrangement of Intra-membrane Particles?

Freeze-fracturing revealed that intramembrane particles of the ghosts loaded with anti-spectrin antibody along with BSA were randomly distributed, while Nicolson and Painter (17) reported that the antibody could induce clustering of surface anionic sites of the erythrocyte membrane (practically identical to glycophorin). This apparent discrepancy can be explained as follows. (1) The conditions, at which the antibody was introduced into ghosts and then bound to spectrin, were quite different with each other. Thus inclusion of the antibody and subsequent binding to spectrin were performed throughout in isotonic medium in the present study, but they included the antibody within ghosts in hypotonic medium and thereafter ghosts were incubated at 37° for 30 min to induce clustering of surface anionic sites. These operations adopted by them may, as pointed out by Elgsaeter and Branton (15), significantly impair the spectrin meshwork and presumably remove certain amount of spectrin from the cell membrane, thus increasing the mobility of membrane glycoproteins and allowing them to aggregate by the action of the antibody. (2). The ghosts used in the present study was prepared by the method of Dodge et al. (56), washed once, then immediately subjected to loading with the antibody and BSA, while ghosts used in their studies were extensively sashed with hypotonic medium (3-5 times) and further incubation

in isotonic saline overnight to allow to reseal, thus causing significant damages in the integrity of spectrin meshwork. (3) The optimal concentration of the antibody to cause extensive clustering of surface anionic sites were 0.25-1.0 mg/ml and further increase in the concentration of added antibody (>5 mg/ml) rather resulted in dispersed distribution of the anionic sites. The random distribution of intramembrane particles on the ghosts loaded with the antibody (5 mg/ml) along with BSA is , therefore, apparently consistent with their observation. But lower concentrations of the antibody (0.25-1.0 mg/ml) could neither induce clustering of intramembrane particles under isotonic conditions (data not shown), suggesting that any concentration of the antibody can not affect the random distribution of intramembrane particles unless spectrin was partially removed from the erythrocyte membrane under hypotonic condition.

Can Other Agents which Specifically Interact with Spectrin Affect the Fusion Reaction?

There is some evidence suggesting that muscle actin ineracts with spectrin (64-67). Pinder et al. have shown that spectrin could initiate the polymerization of muscle G-actin and furthermore that the ability of spectrin to induce actin polymerization is regulated by phosphorylation of one of spectrin subunits, Band II (65,67). Tilney and Detmers also showed that spectrin could bind to F-actin and thereby increase the viscosity of F-actin. (64). Similar observation has been obtained using flow birefringence technique (66). Thus, inhibitory effect of included G-actin, which was presumably converted into F-actin during the resealing process, on the fusion reaction of erythrocyte ghosts might be due to the association of F-actin with spectrin meshwork.

Recently Birchmeier and Singer have shown that ATP-dependent shape changes of erythrocyte ghosts, in which spectrin meshwork also plays a critical part, was inhibited by muscle G-actin (68), although effective concentrations of actin were one order lower than that needed for inhibition of the fusion reaction. They discussed that since half maximal inhibition occurred at 20 $\mu\text{g/ml}$ and this value was several times lower than those reported to be required to form F-actin under optimal conditions (56 $\mu\text{g/ml}$ ref. 75), actin may exert its inhibitory effect as G-actin. In the case of the fusion reaction, more than 1.0 mg/ml of muscle G-actin was required to obtain extensive inhibition so that included G-actin was almost polymerized into F-actin. Therefore, it may be reasonable to consider that inhibition of the fusion reaction by muscle actin may be caused by association of F-actin with spectrin meshwork, which may stabilize it against viral action. On the contrary, slight stimulation of the fusion reaction by DNAase I may be due to the extraction of endogenous actin from the erythrocyte membrane which results in slight destabilization of spectrin meshwork.

Control Mechanism of Envelope Fusion Is Different from That of Cell-Cell Fusion. It is generally accepted that virus-induced cell fusion reaction is constituted of two different membrane fusion phenomena, i.e., envelope fusion and cell-cell fusion, which seems to be independently regulated by different molecular mechanisms and therefore can be separated under certain conditions. Indeed, anti-spectrin antibody inhibits cell-cell fusion whereas it could not affect envelope fusion. Furthermore, as shown in the preceding paper, the efficiency of envelope fusion is almost the same between non-fusible ghosts and fusible BSA-loaded ghosts. Recently, Lalazar et

al. have shown that fusion ability of erythrocyte ghosts prepared by rapid hemolysis could be restored by the treatment of sulfhydryl-blocking agents but such treatment did not affect the extent of virus-induced lysis (76), suggesting that the efficiency of envelope fusion was not influenced by the treatment. In the case of the fusion of Ehrlich ascites tumor cells, separation between envelope and cell-cell fusion could be observed under the conditions where cell-cell fusion was selectively inhibited by inhibitors for oxidative phosphorylation or cytochlasin D (78).

There is some evidence indicating that envelope fusion is critically dependent on the function of viral glycoprotein, F, and selective removal of F glycoprotein by mild trypsin digestion results in the failure to undergo envelope fusion whereas the trypsinized virus can still agglutinate cells (79). Since envelope fusion is an essential step to inject viral genetic materials into cells, it seems reasonable that the efficiency of envelope fusion is strictly dependent on viral factors and to lesser extent on cellular surface modulation. In other words, viral envelope (or F glycoprotein) may have a specialized ability to fuse with any cell membrane which carries specific receptor. The molecular mechanism(s) of envelope fusion, however, has not yet been clear and therefore one of the most important problems to be solved.

In contrast to envelope fusion, cell-cell fusion seems to be regulated in much complicated manner by various cellular factors which contain extracellular surface charges, fluidity of membrane lipid matrix (80), colloidal osmotic gradient across the cell membrane and lateral mobility and topography of membrane glycoproteins and intramembrane particles (31-40,46,47,80). As shown in the pre-

sent paper, mobilization and rearrangement of intramembrane particles plays a most critical part in cell-cell fusion of intact erythrocytes and their ghosts. Although it is still ambiguous whether such mobilization and rearrangement of intramembrane particles is also required for the virus-induced fusion of other nucleated cells, here is an indication that native microfilaments may be necessary for the fusion of Ehrlich ascites tumor cells (78), suggesting, together with the requirements for metabolic energy and calcium ion (81), that similar redistribution of membrane glycoproteins, whose lateral mobility is believed to be regulated by intracellular microfilaments and microtubules (16,82), may be induced during cell-cell fusion. Studies on the control mechanism of virus-induced fusion reaction of both erythrocytes and nucleated cells may, therefore, provide a useful information on the mechanisms of transmembrane control of membrane topography.

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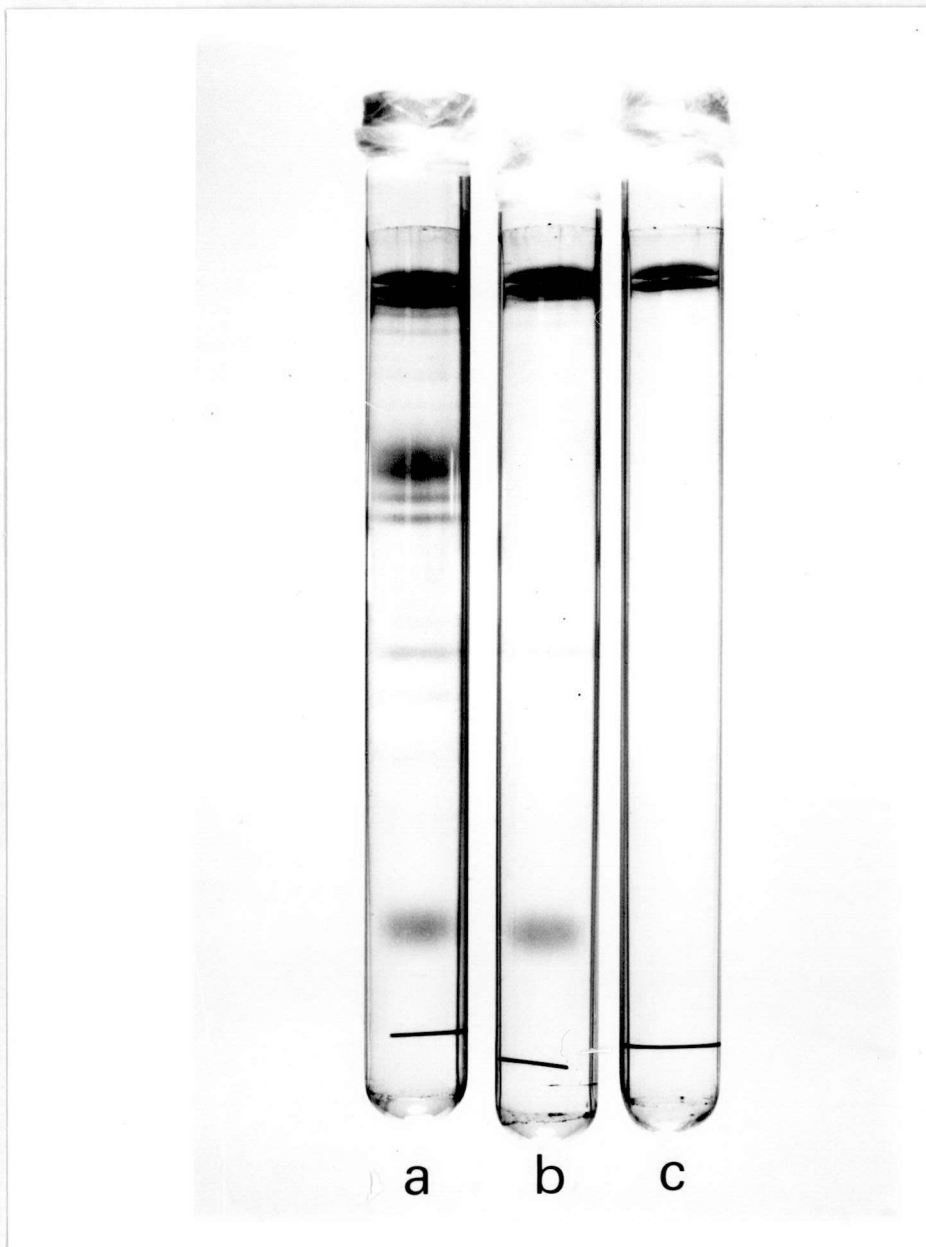


Fig. 1. SDS-polyacrylamide gel electrophoresis of erythrocyte membrane (a), low ionic strength extract of erythrocyte ghosts (b), partially purified spectrin by isoelectric precipitation (c). 5.6 % polyacrylamide gels. Partially purified spectrin, which was coupled with CNBr-activated Sepharose 4B for the purification of anti-spectrin antibody, was almost devoid of any other membrane polypeptides except a faint contamination of Band V.

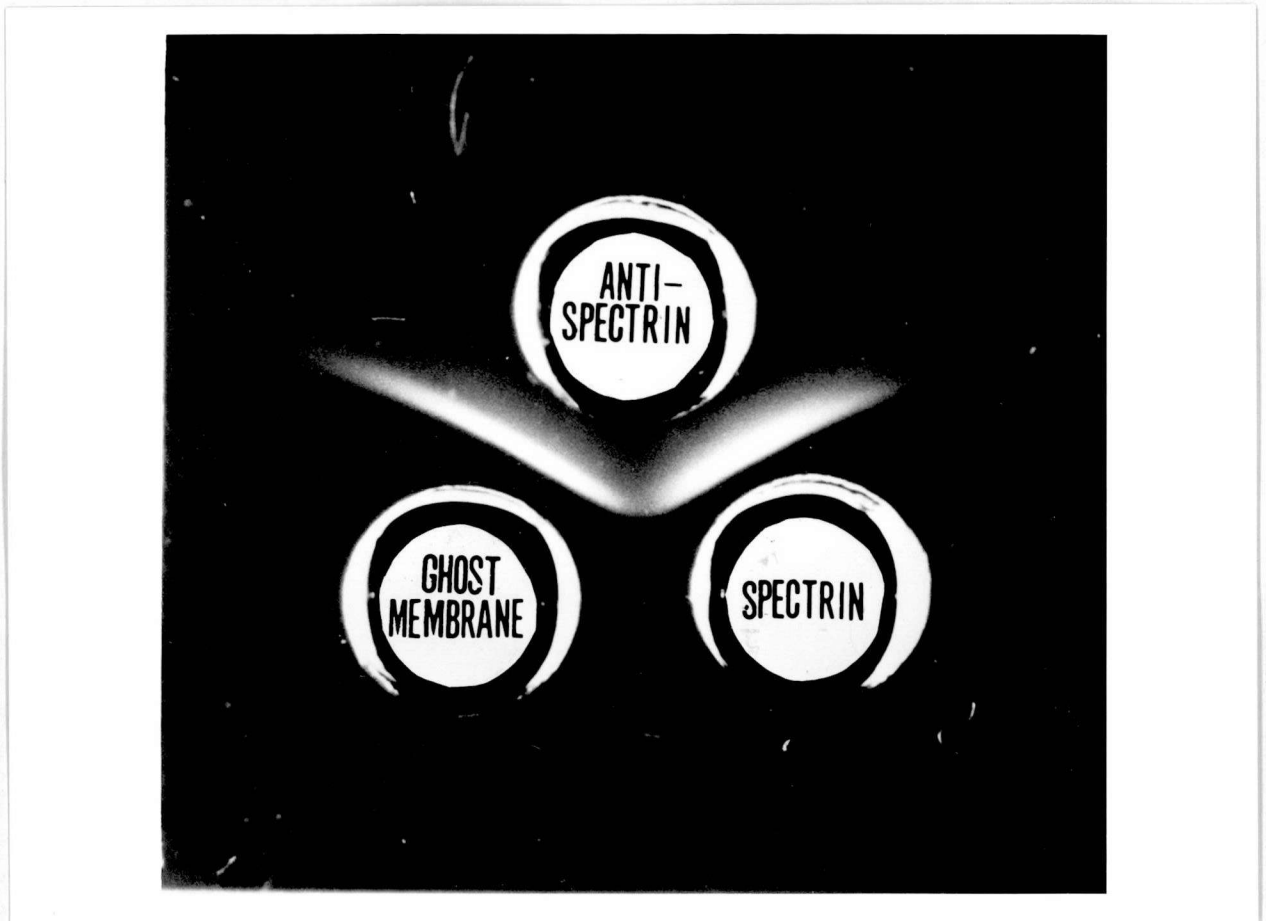


Fig. 2. Double immunodiffusion test of purified anti-spectrin antibody. The Ouchterlony plate was made of 1 % agarose containing 10 mM DOC, 10 mM Tris·HCl, 0.1 mM EDTA, 50 mM NaCl, pH 8.0. Center well (top) was purified anti-spectrin antibody; right (below), purified spectrin; left (below), whole erythrocyte membrane solubilized with 10 mM DOC. Note that anti-spectrin formed single precipitin band with both whole erythrocyte membrane and purified spectrin.

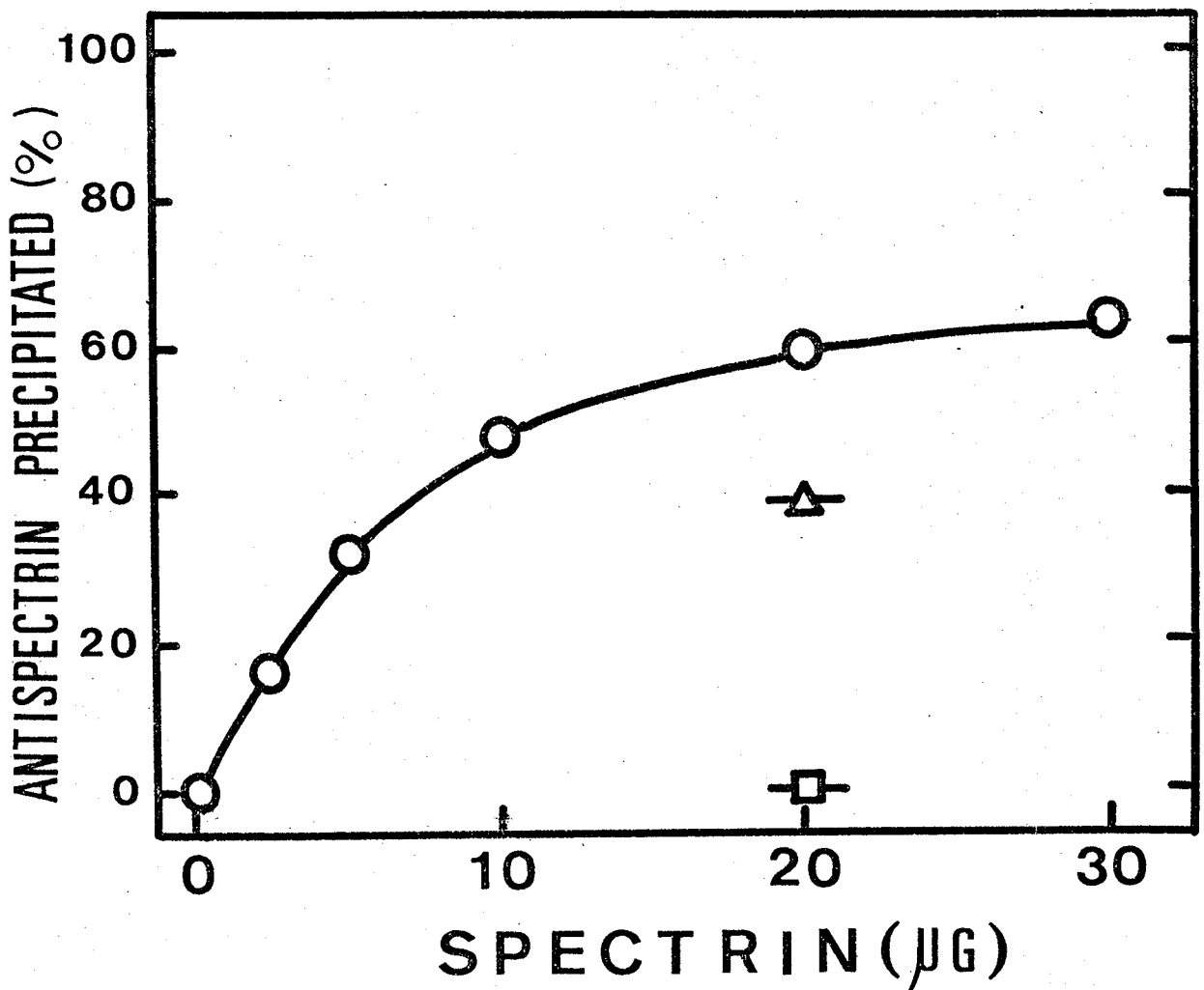


Fig. 3. Quantitative antigen-antibody precipitation curve. Increasing amount of purified spectrin was added to ^{125}I -labeled anti-spectrin antibody (227 μg) in 0.5 ml of 20 mM Tris-HCl (pH 7.4), then incubated at 37° for 30 min and further at 4° overnight. Precipitated antigen-antibody complex was twice washed with 20 mM Tris buffer and the radioactivity of the precipitate^(-O-) was determined with well-type gamma scintillation spectrometer. Fab fragments (\triangle , 166 μg ; \square , 335 μg) were first preincubated with spectrin at 37° for 15 min and then mixed with anti-spectrin antibody.

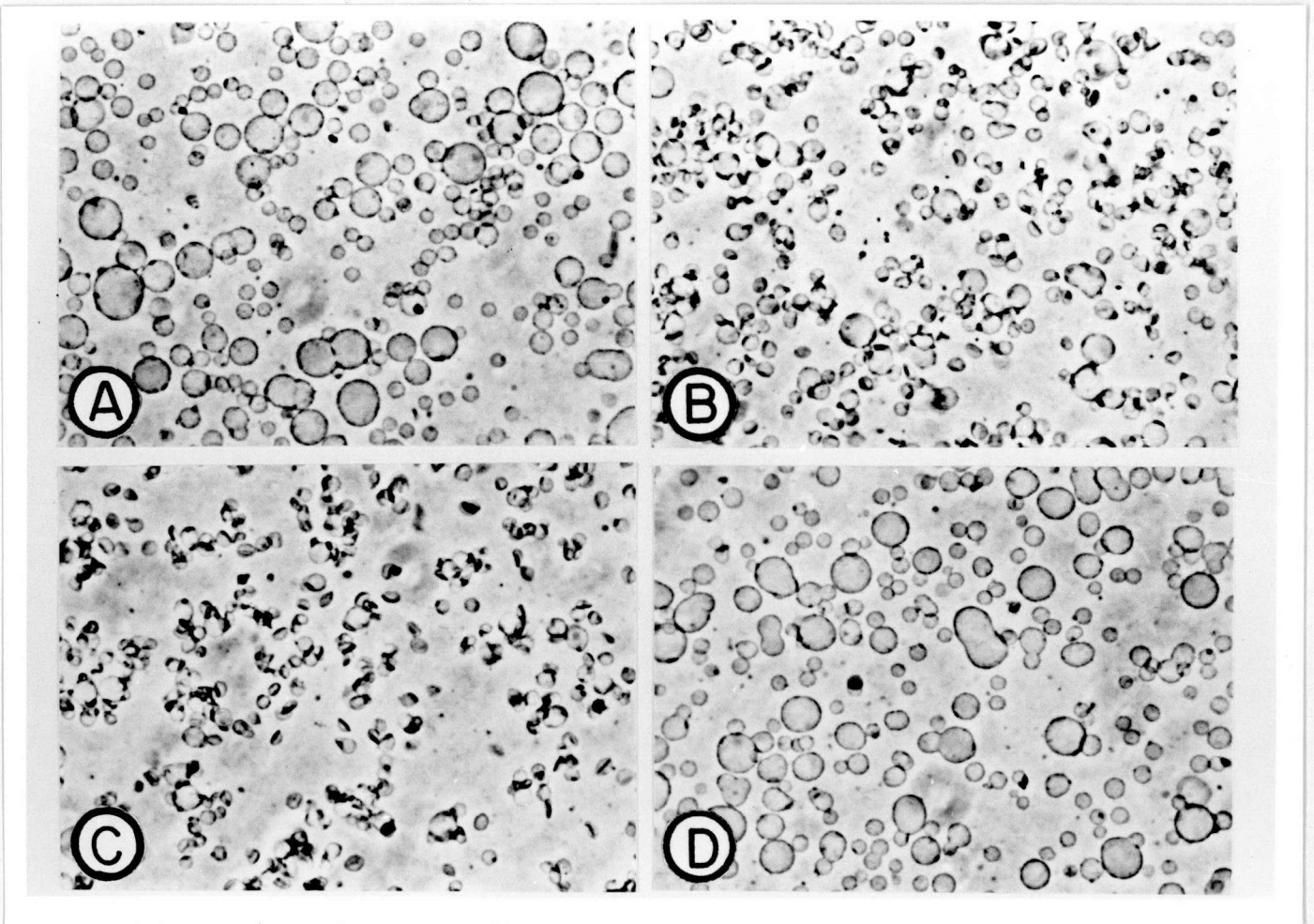


Fig. 4. Effect of anti-spectrin antibody on the virus-induced fusion of erythrocyte ghosts. Ghosts were loaded with 5 % BSA containing the following proteins of indicated concentrations by the method described in "Materials and Methods"/ (A), Control immunoglobulin (4 mg/ml); (B) and (C), anti-spectrin antibody (1 and 4 mg/ml, respectively); (D), Fab fragments prepared from the antibody (4 mg/ml). These ghosts were agglutinated by HVJ (10,000 HAU/ml) for 15 min in an ice bath and then incubated at 37° for 30 min. Phase microscopy, x350.

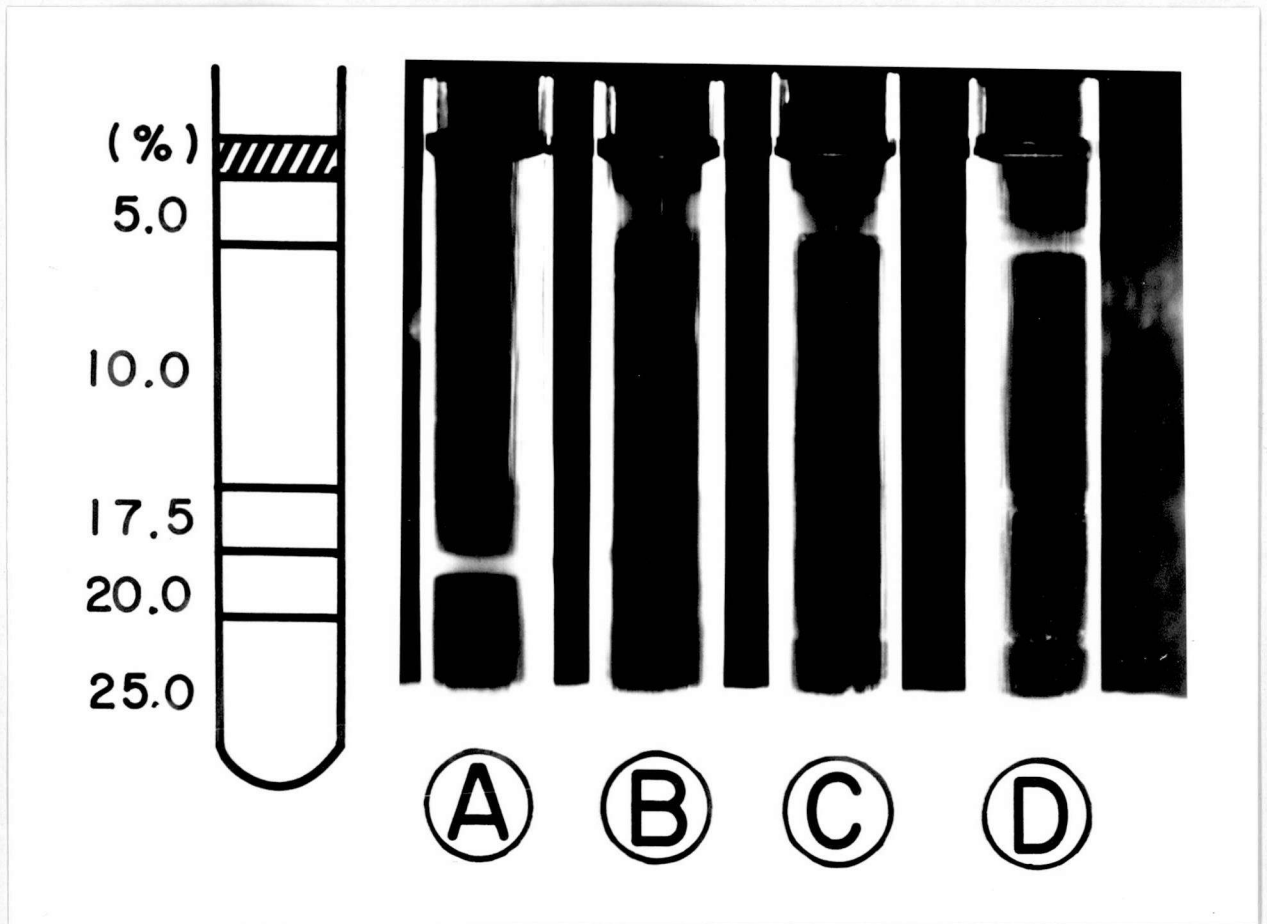


Fig. 5. Dextran T 70 discontinuous density gradient centrifugation. Discontinuous gradient of dextran T 70 (dissolved in TBS) was formed in the centrifuge tubes of Spinco SW-41 rotor, as depicted left. Samples applied on the gradients were (A) unsealed ghosts; (B) BSA-loaded ghosts; (C) BSA and control immunoglobulin (4 mg/ml)-loaded ghosts; (D) BSA and anti-spectrin antibody (4 mg/ml)-loaded ghosts. Samples were centrifuged for 3.5 h at 36,000 rpm (Spinco SW-41) at 4°.

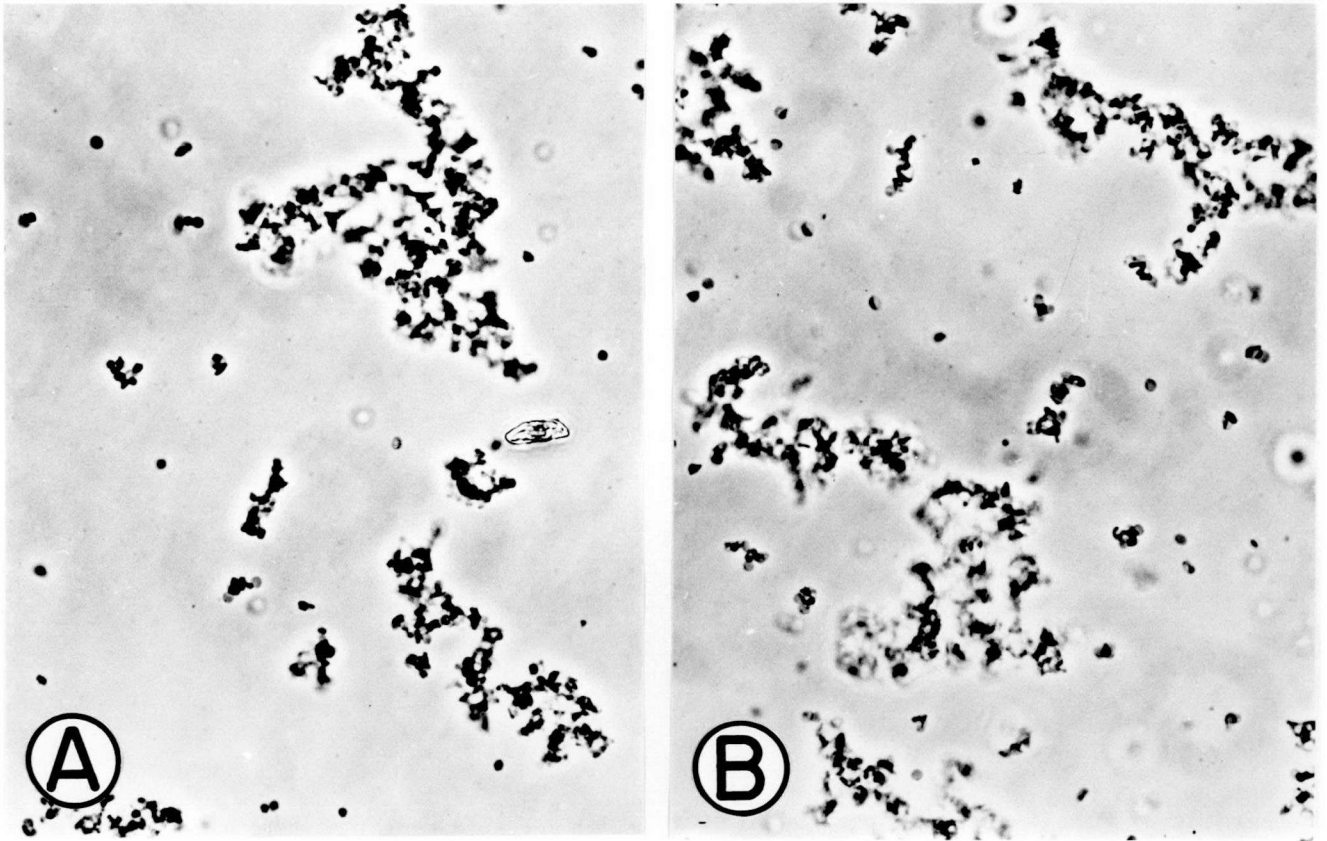


Fig. 6. Effect of anti-spectrin antibody on the virus-induced agglutination of ghosts. Ghosts loaded with BSA and either (A) control immunoglobulin (4 mg/ml) or (B) anti-spectrin antibody (4 mg/ml) were prepared as described in "Materials and Methods". Ghosts were agglutinated by HVJ (10,000 HAU/ml) for 15 min in an ice bath.

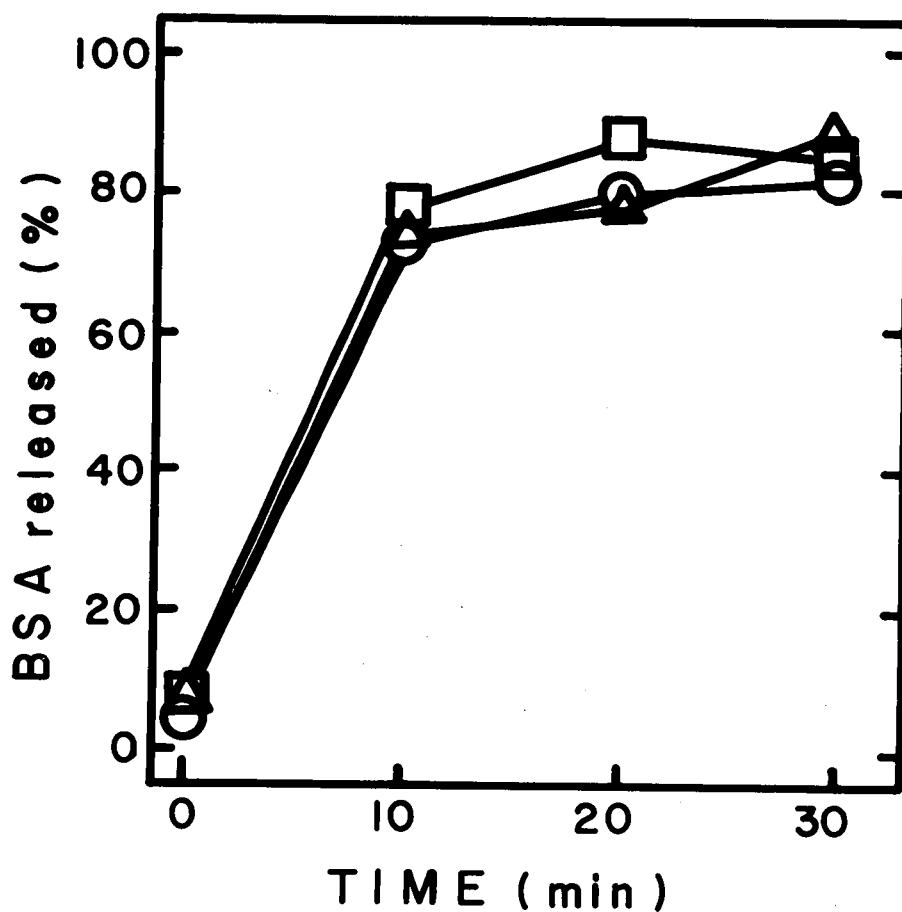


Fig. 7. Effect of anti-spectrin antibody on the virus-induced leakage of included BSA. Ghosts loaded with [125 I]BSA and the following proteins were prepared as described in "Materials and Methods". (○), No addition; (△), control immunoglobulin (4 mg/ml); (□), anti-spectrin antibody (4 mg/ml). Ghosts were first agglutinated by HVJ (10,000 HAU/ml) for 15 at 0° then incubated at 37° for different periods. The amount of released BSA was determined as described in "Materials and Methods".

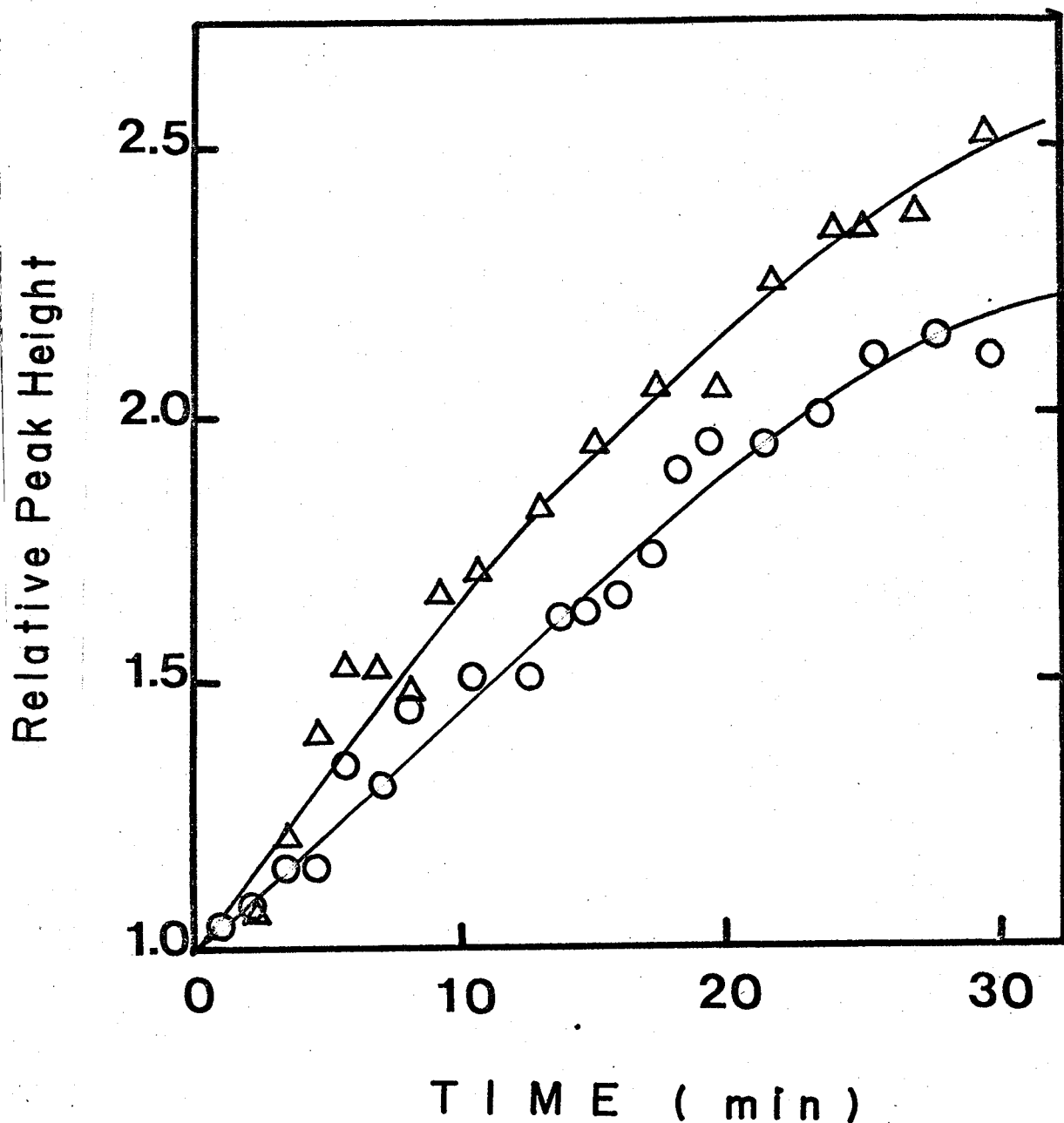


Fig. 8. Effect of anti-spectrin antibody on the transfer of PC* from HVJ* to the erythrocyte membrane. Ghosts loaded with BSA and either (A) control immunoglobulin (4 mg/ml) ^(-Δ-) or (B) anti-spectrin antibody (4 mg/ml) ^(-O-) were prepared as described in "Materials and Methods". These ghosts were agglutinated by a mixture of HVJ* (300 HAU/ml) and non-labeled HVJ (5,200 HAU/ml) for 15 min in an ice bath, then lightly centrifuged at 2,000 rpm 5 min. The packed aggregates were taken into a quartz capillary tube and the changes of ESR spectrum during the subsequent incubation at 37° were followed. Relative increase in central peak height was plotted against incubation time.

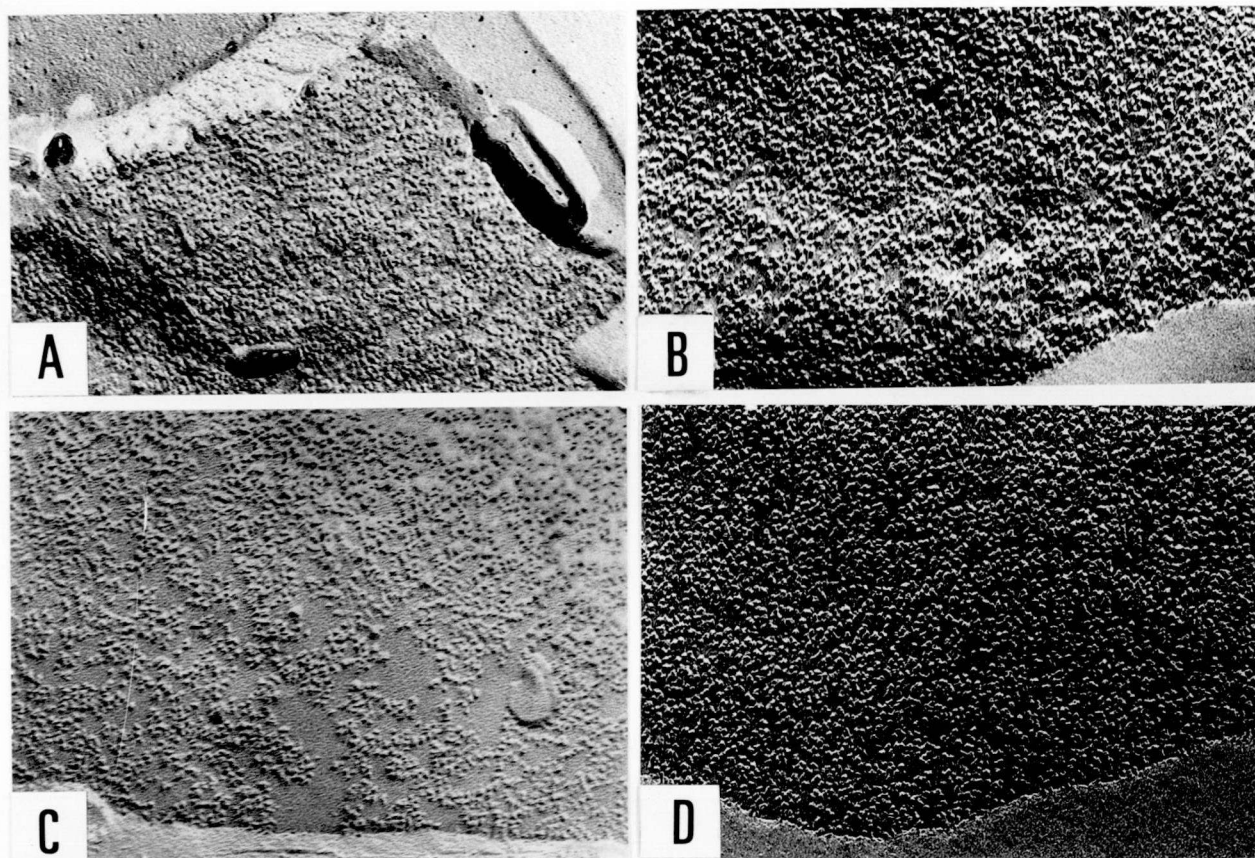


Fig. 9. Effect of anti-spectrin antibody on the distribution of intramembrane particles. (A), Protoplasmic surface (PF) of ghosts loaded with BSA and control immunoglobulin (5 mg/ml). (B), PF of ghosts loaded with BSA and anti-spectrin antibody (5 mg/ml). (C), The same as (A) except that ghosts were reacted with HVJ (8,000 HAU/ml) at 37° for 30 min. Note that rearrangement of intramembrane particles took place. (D), The same as (C) except that antibody-treated ghosts were reacted with HVJ (8,000 HAU/ml) at 37° for 30 min. Distribution of the particles was still random even after virus-treatment.

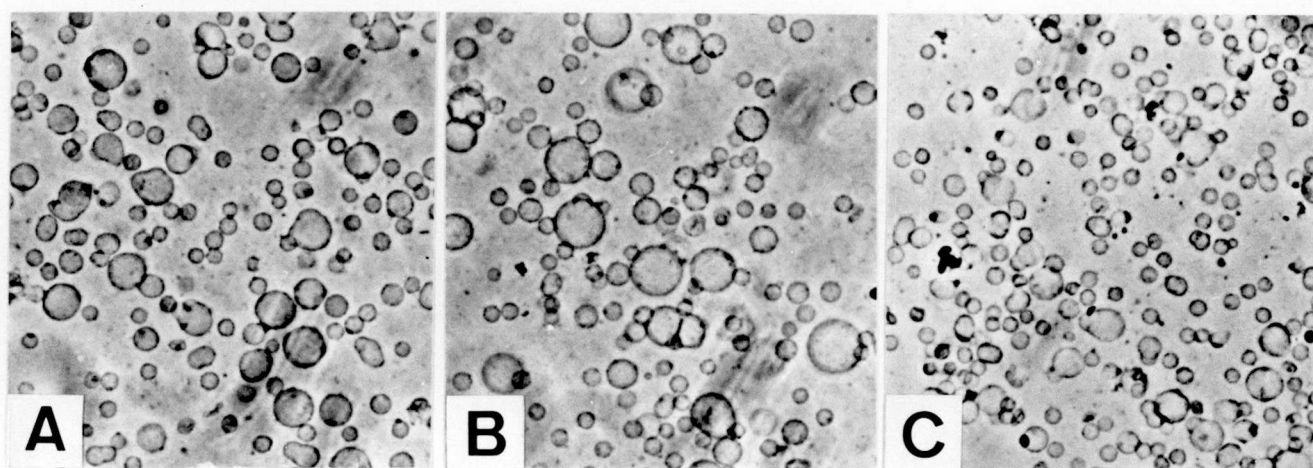


Fig. 10. Effect of externally added anti-spectrin antibody on the fusion reaction of BSA-loaded ghosts. BSA-loaded ghosts were first agglutinated by HVJ (16,000 HAU/ml) for 15 min in an ice bath and then added the following substances and immediately incubated at 30° for 60 min. (A), TBS; (B), control immunoglobulin (4.3 mg/ml); (C), anti-spectrin antibody (4.3 mg/ml): Phase microscopy, x350.

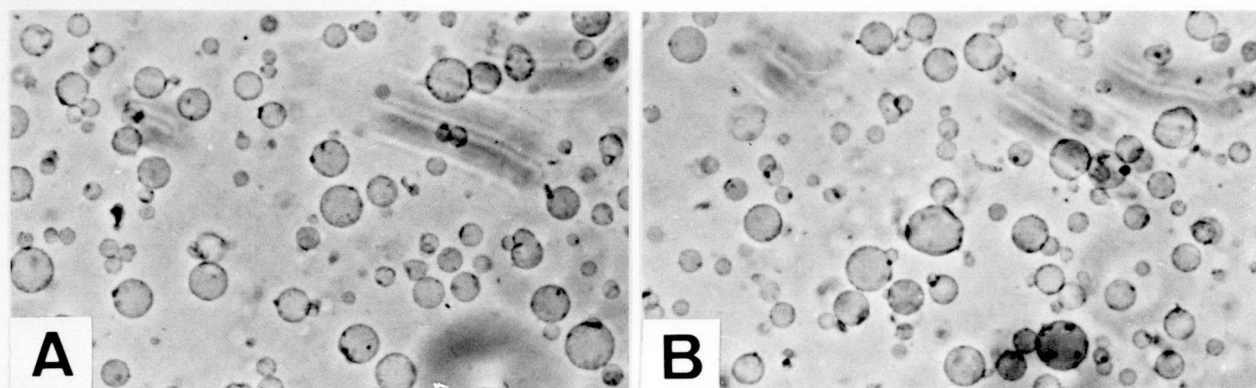


Fig. 11. Reversion of inhibitory effect of externally added antibody by washing before virus-treatment. BSA-loaded ghosts were incubated at 37° for 30 min in the absence (A) or presence (B) of externally added antibody (4.3 mg/ml). These ghosts were washed with TBS and then agglutinated by HVJ (12,000 HAU/ml) for 15 min in an ice bath and subsequently incubated at 37° for 60 min. Note that inhibitory effect of externally added antibody was reversed by washing before the virus-treatment. Phase microscopy, x350.

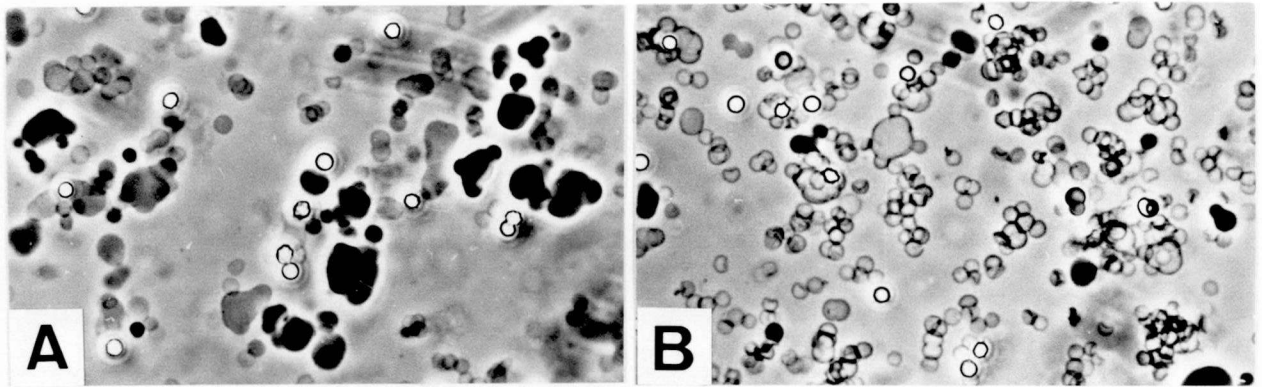


Fig. 12. Effect of externally added antispectrin antibody on the fusion of intact erythrocytes. Intact erythrocytes were first agglutinated by HVJ (1,070 HAU/ml) for 15 min in an ice bath then added either (A) TBS or (B) anti-spectrin antibody (13.7 mg/ml), and subsequently incubated at 30° for 30 min and further at 37° for additional 30 min. Phase microscopy, x350.

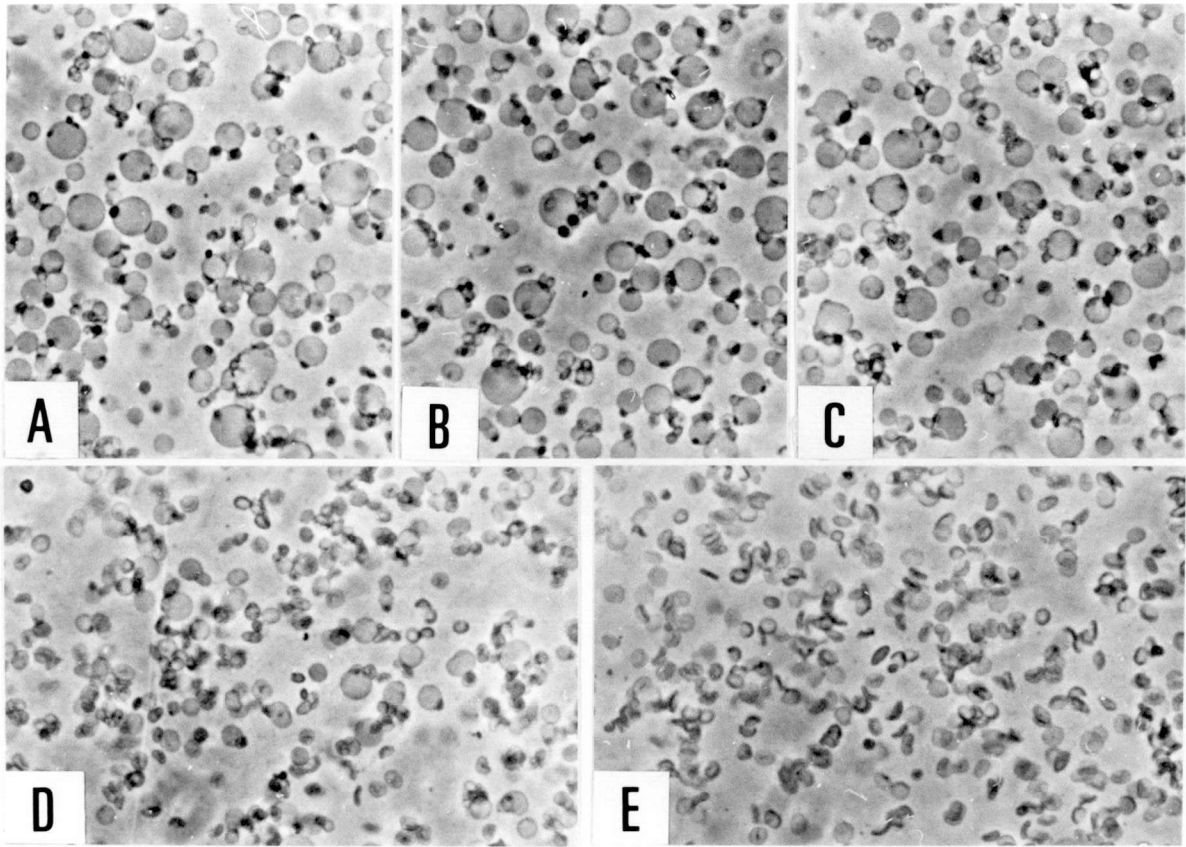


Fig. 13. Effect of muscle actin on the fusion reaction of erythrocyte ghosts. Ghosts loaded with BSA and different concentrations of muscle actin were prepared as described in "Materials and Methods". (A), 0 mg/ml; (B), 0.25 mg/ml; (C), 0.5 mg/ml; (D), 1.0 mg/ml; (E), 2.0 mg/ml. These ghosts were agglutinated by HVJ (6,000 HAU/ml) for 15 min in an ice bath and subsequently incubated for 60 min at 37°. Phase microscopy, x350.

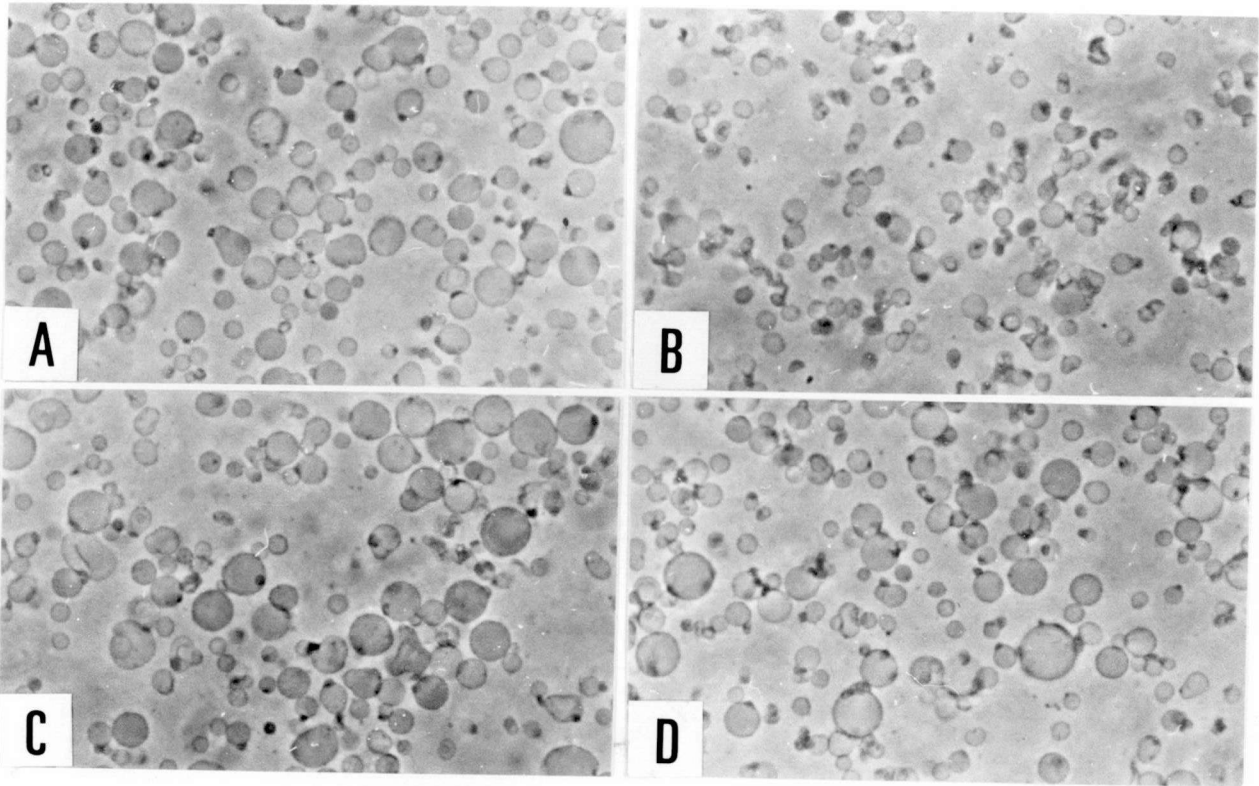


Fig. 14. Antagonistic effect of DNAase I on inhibition of muscle actin on the fusion reaction. Erythrocyte ghosts loaded with BSA and the following proteins were prepared as described in "Materials and Methods". (A), No addition; (B), muscle actin (1 mg/ml); (C), DNAase I (0.96 mg/ml); (d), muscle actin complexed with DNAase I before inclusion within ghosts. These ghosts were agglutinated by HVJ (6,000 HAU/ml) for 15 min in an ice bath and subsequently incubated for 60 min at 37°. Phase microscopy, x350.

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