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Virus and lectin agglutination of erythrocytes: Spin label study of membrane lipid–protein interactions

(Sendai virus/influenza virus/concanavalin A/wheat germ agglutinin/microtubules)

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ABSTRACT **Techniques of spin-label electron spin resonance have been used to probe changes in the structure of the lipid phase of erythrocyte membrane after agglutination by viruses and lectins. When chicken erythrocytes are agglutinated by Sendai and influenza viruses and by the lectins concanavalin A and wheat germ agglutinin, the membrane lipid phase becomes more fluid, as detected by three different lipophilic spin-labeled probes. Colchicine, vinblastine, and tetracaine inhibit the fluidization of chicken erythrocyte membrane by Sendai virus, whereas cytochalasin B has no effect. The effect of colchicine was time dependent, the initial inhibition decreasing with longer preincubation times. Extensive treatment of erythrocytes with proteases or neuraminidase, while not altering the bilayer structure, abolishes the effect of Sendai virus on the erythrocyte membrane, suggesting that a change in the interaction of the receptor protein with the lipid phase occurs upon virus attachment. Glutaraldehyde fixation increased the structural rigidity of the chicken erythrocyte membrane and inhibited the effect of viral agglutination. No change in bilayer structure was observed upon agglutination of human erythrocytes or the isolated plasma membranes of either human or chicken erythrocytes. This result is consistent with the drug sensitivity of the effects of agglutination upon chicken erythrocytes, since human erythrocytes and isolated membranes lack microtubule-like structures.**

Many enveloped viruses such as influenza and parainfluenza viruses have a hemagglutinin, which is a glycoprotein on the outer surface of the membrane-like envelope (1, 2). Since these viruses attach to cellular receptors on the host plasma membrane through the hemagglutinin protein, the agglutination of erythrocytes provides a model for the earliest events in viral infection. Lectins are carbohydrate-binding proteins isolated from various plant and animal sources. These proteins contain multiple binding sites capable of crosslinking and agglutinating cells. Lectins have been used extensively as probes of surface structure in a wide variety of applications (3). The receptors on the erythrocyte membrane for many of these agglutinins are glycoproteins that penetrate deeply into the membrane lipid bilayer. Some of these receptors are exposed at the inner surface of the membrane (ref. 4 and refs. cited therein). Since these proteins interact extensively with the lipid phase of the membrane, binding of agglutinins, such as lectins or viruses, might be expected to cause changes in these lipid–protein interactions. We have investigated this hypothesis using techniques of spin label electron spin resonance (ESR) to probe the structure of the lipid phase of erythrocyte membrane.

Our results show that when chicken erythrocytes are agglutinated by either viruses or lectins, the fluidity of the membrane lipid bilayer increases. However, no change is observed in the fluidity of the membrane lipid phase of the human erythrocyte upon attachment of viruses or lectins. The structural changes in the membrane lipid phase of the chicken erythrocyte appear to be due to the action of systems that resemble microtubules in their drug sensitivity. This dependence upon microtubule-like systems may also account for the observed species differences.

Abbreviation: ESR, electron spin resonance.

MATERIALS AND METHODS

Chemicals. Concanavalin A was purchased from Pharmacia Co. Colchicine, vinblastine, tetracaine, and bovine serum albumin (less than 0.01% fatty acid) were purchased from Sigma Chemical Co. Trypsin treated with L-(1-tosylamido-2-phenyl)ethylchloromethylketone was obtained from Worthington, and neuraminidase from *Vibrio cholerae* was purchased from Behring Diagnostics, Somerville, N.J. The stearic acid derivative spin labels, C_5 [2-(3-carboxyl-propyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl] and C_{16} [2-(14-carboxyl-tetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl], were obtained from Syva, Palo Alto, Calif. The 5N10 spin label (2-butyl, 2-pentyl, 4,4-dimethyl-*N*-oxyl oxazolidine) was synthesized as described (5) and purified by column chromatography on silicic acid. The purity was ascertained by thin layer chromatography on silica gel in benzene:ether (7:1 vol/vol) and petroleum ether:acetone (9:1 vol/vol).

Virus and Cells. The WSN strain of influenza A_0 virus and Sendai virus cultivated in embryonated hen eggs were grown in the Madin-Darby line of bovine kidney cells (6, 7). Viruses were harvested and purified as described (8). Hemagglutination titrations were performed as described (7).

Spin Labeling of Erythrocytes. Erythrocytes were obtained from freshly drawn heparinized human and chicken blood by centrifugation at 1000 × *g* for 10 min, followed by aspiration of the plasma and buffy coat. The cells were washed four times with cold phosphate-buffered saline. Spin-labeled erythrocyte ghosts were prepared from labeled human erythrocytes (9). Plasma and nuclear membranes were isolated from chicken erythrocytes disrupted in a French pressure cell and purified on a 30–70% (wt/vol) linear sucrose gradient (10). The intact cells and membrane preparations were labeled with C_5 and C_{16} spin labels, using a spin label-bovine serum albumin complex (8). A 50% (vol/vol) suspension of labeled erythrocytes or membranes in phosphate-buffered saline was added to an equal volume of the buffer containing virus (2000 hemagglutination units per ml) or lectins (500 μg/ml), placed in a capillary sealed at one end, and centrifuged for 5 min in a clinical table-top centrifuge. No detectable hemolysis occurred either from label incorporation or from agglutination. Erythrocytes were judged to be free of adsorbed spin label-bovine serum albumin complex by the absence of the distinctly different spectrum of protein-bound spin label in C_{16}-labeled erythrocytes (11).

To label erythrocytes with 5N10 spin label, an ethanol solution of 5N10 (0.1 M) was evaporated to dryness in the bottom of a test tube with a thin stream of dry nitrogen. A 25% (vol/vol) suspension of erythrocytes in phosphate-buffered saline was added to give a final concentration of 5N10 of 5 mM. The

Table 1. ESR spectral splitting of the C_5 label in erythrocyte membranes after agglutination

Agglutinin	$2A'_{zz}$ (gauss)			
	Chicken erythrocyte	Human erythrocyte	Human erythrocyte ghosts	Chicken erythrocyte plasma membrane
None	58.2 ± 0.09 (10)*	58.1 ± 0.07 (5)*	58.0	58.1
Sendai virus	57.0 ± 0.2 (11)*	58.3 ± 0.09 (5)*	58.0	57.9
Influenza virus	56.9	58.3		
Concanavalin A	57.0	58.0†		
Wheat germ agglutinin	56.9	58.1		

* Mean ± standard error of the mean with number of experiments given in parentheses for experiments repeated five or more times. All other data in this and following tables are the average of two to five independent experiments. In general, differences in $2A'_{zz}$ greater than ±0.3 gauss can be detected by superposition of spectra.

† Cells weakly agglutinated.

sample was incubated at room temperature for 1 hr. An aliquot of this preparation was added to an equal volume of either virus or buffer. The sample was placed in a capillary sealed at one end and centrifuged in a clinical centrifuge for exactly 5 min. The supernatant was aspirated. This technique gave reproducible partitioning of spin label between aqueous and lipid phases.

ESR spectra were recorded at room temperature with a Varian E-12 spectrometer, interfaced to a Texas Instruments 980A computer (12). The spectra presented were recorded at 24°.

RESULTS

Fig. 1 and Table 1 show the effect of agglutination on the ESR spectrum of the C_5 stearic acid derivative spin label incorporated into the membrane of chicken erythrocytes. The C_5 spin label probes the lipid bilayer in the region near the glycerol backbone of the membrane phospholipids (13). When chicken erythrocytes are agglutinated by Sendai virus grown in Madin-Darby bovine kidney [which has hemagglutinin and neuraminidase activities but lacks cell fusing or hemolyzing activities (7)], by influenza virus, or by the lectins concanavalin A and wheat germ agglutinin, the fluidity of the lipid phase appears to increase, as revealed in a small, but easily detectable decrease in the distance between the outermost peaks of the spectrum ($2A'_{zz}$), defined in Fig. 1 (14, 15). The interior hydrocarbon region of the bilayer is probed by the C_{16} stearic acid derivative spin label (13). The ESR spectrum of C_{16}-labeled chicken erythrocytes agglutinated by Sendai virus shows that the peak marked by an arrow in Fig. 2 appears to have moved toward the solid vertical line. This indicates that the fluidity increase observed with the C_5 label is also observed in the interior of the bilayer.

The increase in fluidity of the chicken erythrocyte bilayer upon agglutination was also detected with a 5-nitroxide derivative of decane (5N10, Fig. 3) which partitions between aqueous and lipid phases (5). This partitioning splits the highfield peak (Fig. 3). The quantities h_L and h_P (defined in Fig. 3) correspond in a first approximation to the fraction of label in the lipid phase and aqueous phase, respectively. The solubility of the probe in the lipid phase increases as the bilayer fluidity increases. The decrease in h_P/h_L of 5N10-labeled chicken erythrocytes after agglutination (Fig. 3) confirms the increase in fluidity detected with the C_5 and C_{16} spin labels. Possible spectral effects resulting from addition of viral lipid, either by fusion with erythrocytes or by partitioning of spin labels between virus and erythrocyte bilayers, are negligible, since the amount of viral lipid added is small compared to that of the erythrocytes. Also, the viral envelope bilayer is more rigid than the erythrocyte bilayer (8), so that the changes observed are the opposite of results expected from either fusion or repartitioning of labels.

In contrast to the results obtained with chicken erythrocytes, no significant structural changes in the lipid bilayer were detected upon agglutination of human erythrocytes with either viruses or lectins (Table 1). The spectral splitting ($2A'_{zz}$) of the C_5 spin label, and thus the fluidity of the probe environment

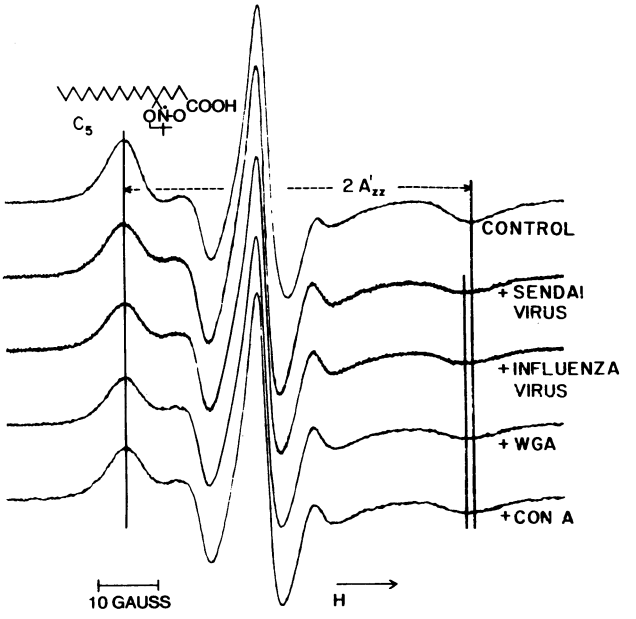

FIG. 1. Effect of agglutination of chicken erythrocytes by viruses or lectins. Vertical lines are drawn through the extrema of the outermost peaks of the spectrum. The splitting of the outermost peaks ($2A'_{zz}$) is defined for the control spectrum (unagglutinated erythrocytes). Spectra of agglutinated cells differ from those of controls in other parameters besides $2A'_{zz}$. The relative peak heights of the extreme high- and lowfield peaks are decreased upon agglutination; the highfield side of the lowfield peak does not return to baseline in spectra of agglutinated cells as it does in controls; and there is an increased resolution of the first peak on the lowfield side of the highfield peak in the spectra of agglutinated cells. All of these spectral changes are consistent with a decrease in $2A'_{zz}$ upon agglutination, indicating an increase in bilayer fluidity. Con A, concanavalin A; WGA, wheat germ agglutinin.

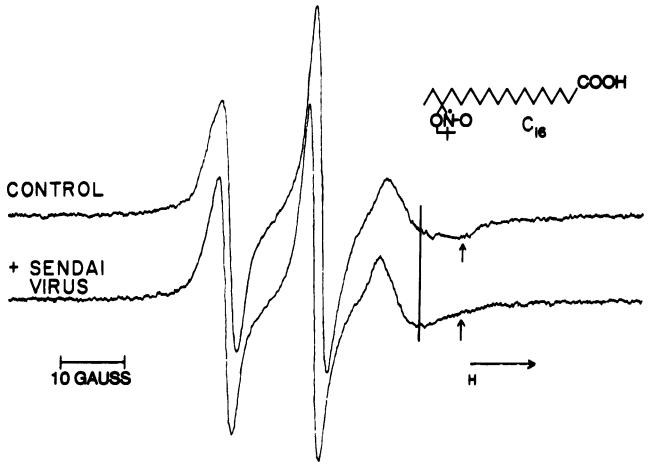

FIG. 2. ESR spectra of C_{16} spin label in chicken erythrocyte membranes. Each spectrum is a time average of four repetitive scans. Arrows and vertical line indicate positions of minima in the highfield peak of the spectrum of control and virus-agglutinated cells, respectively.

in the human erythrocyte, is practically identical to that in the chicken erythrocyte. Occasionally, a small decrease in membrane fluidity was noted upon agglutination of C_5-labeled human erythrocytes, amounting to an increase in $2A'_{zz}$ of 0.5 gauss or less. Agglutination of human erythrocyte ghosts or isolated plasma membranes from chicken erythrocytes produces no detectable change in membrane fluidity (Table 1).

Since the agglutinins used in these experiments bind primarily to glycoproteins on the erythrocyte surface (see ref. 3), the change in structure of the lipid phase of the chicken erythrocyte plasma membrane upon agglutination suggests that changes in membrane lipid-protein interactions occur after agglutination. Table 2 illustrates the effect of digesting virus receptors on the erythrocyte surface with either proteases or neuraminidase. Pretreatment of chicken erythrocytes with trypsin, Pronase, or neuraminidase abolishes the bilayer fluidity increase that accompanies the addition of Sendai virus. None of these enzymes alone has a detectable effect on the fluidity

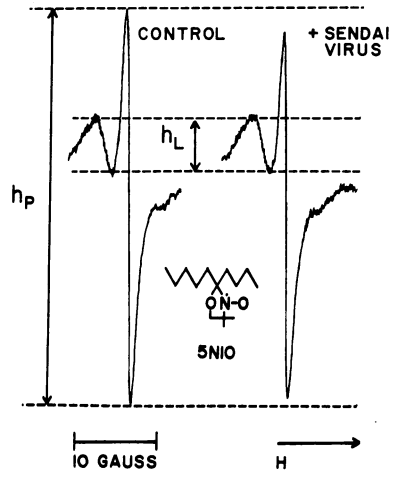

FIG. 3. ESR spectra of highfield peak of 5N10 spin label in chicken erythrocyte membranes. Final concentration of 5N10 was 2.5 mM in a sample consisting of a 12.5% (vol/vol) suspension of chicken erythrocytes with or without Sendai virus (1000 hemagglutination units/ml, final concentration). h_L, a measure of the amount of label in the membrane lipid phase, is the same for both spectra. h_P, corresponding to label in the aqueous phase, is defined for the control spectrum.

Table 2. Effect of enzymatic digestion of erythrocyte surface upon ESR spectral changes after agglutination of C_5-labeled erythrocytes

Treatment	$2A'_{zz}$ (gauss)	
	Chicken erythrocyte	Human erythrocyte
None	58.2	58.1
+ Sendai virus	57.0	58.3
Pronase*	58.0	58.3
+ Sendai virus	57.9	58.1
Trypsin*	58.2	58.0
+ Sendai virus	58.7	58.0
Neuraminidase†	58.8	58.1
+ Sendai virus	58.2	58.1

* A 50% suspension of labeled erythrocytes in phosphate-buffered saline incubated with Pronase or trypsin (500 μg/ml final concentration) at 37° for 2.5 hr, then washed twice with the buffer.
† A 20% suspension of labeled erythrocytes in phosphate-buffered saline (pH 5.5) incubated with *Vibrio cholerae* neuraminidase (200 units/ml final concentration) at 37° for 2.5 hr, then washed twice with phosphate-buffered saline (pH 7.4).

of the membrane bilayer of either chicken or human erythrocytes. Thus, specific attachment of virus to glycoprotein receptors is required for the agglutination induced fluidization of the chicken erythrocyte bilayer.

C_5-labeled erythrocytes were fixed with glutaraldehyde prior to the addition of virus to test whether possible movements of membrane proteins could account for the observed spectral changes. As seen in Table 3, fixation of either human or chicken erythrocyte proteins greatly increased the spectral splitting of the C_5 spin label probe, indicating a large increase in the rigidity of the bilayer. Thus, prevention of membrane protein lateral mobility markedly increases the rigidity of the membrane bilayer. These results with red cells contrast with the lack of effect of glutaraldehyde fixation on the fluidity of the lipid phase in protein-free liposomes and in nerve from *Homarus americanus* walking leg muscle (16) and in the envelope of Rauscher murine leukemia virus (F. R. Landsberger and R. W. Compans, unpublished results). Sendai virus agglutinated fixed erythrocytes but did not change the C_5 spectrum from that of fixed erythrocytes alone. The interpretation is complicated by the large effect of glutaraldehyde fixation itself.

Drugs that interact with cytoplasmic fibers such as microfilaments and microtubules affect some forms of membrane protein mobility (17–19). Since it might be postulated that lateral movement of membrane proteins may account for the

Table 3. Effect of glutaraldehyde fixation upon ESR spectral changes after agglutination of C_5-labeled erythrocytes

Treatment	$2A'_{zz}$ (gauss)	
	Chicken erythrocyte	Human erythrocyte
None	58.2	58.1
Sendai virus	57.0	58.3
Glutaraldehyde*	63.2	63.2
Glutaraldehyde + Sendai virus	63.1	63.4

* Erythrocytes suspended in 1% glutaraldehyde in phosphate-buffered saline to give a 20% suspension and incubated at room temperature for 45 min, then washed twice with the buffer.

Table 4. Effect of drugs that disrupt cytoplasmic fibers upon ESR spectral changes after agglutination of C_5-labeled erythrocytes

Treatment*	$2A'_{zz}$ (gauss)	
	Chicken erythrocytes	Human erythrocytes
None	58.2	58.1
+ Sendai virus	57.0	58.3
Cytochalasin B (10 μg/ml)	58.4	57.9
+ Sendai virus	56.2	58.5
Colchicine (400 μg/ml)	58.3	58.0
+ Sendai virus	58.2	58.2
Vinblastine (90 μg/ml)	58.2	58.4
+ Sendai virus	58.9	58.4
Tetracaine (500 μg/ml)	58.3	58.3
+ Sendai virus	58.6	58.3

* A 50% suspension of labeled erythrocytes in phosphate-buffered saline was incubated with drugs at indicated concentrations at 37° for 15 min.

fluidity changes seen in chicken erythrocytes, these drugs were tested for their possible effects on the agglutination-induced increase of membrane fluidity. In concentrations as low as 1 μM, colchicine prevents the increase in fluidity that accompanies agglutination (Table 4). Similar results were obtained with vinblastine and with the local anesthetic, tetracaine. Cytochalasin B did not inhibit the fluidity increase accompanying virus attachment. None of these drugs produces significant changes in bilayer fluidity by themselves at the concentrations used. These results suggest that the increase in lipid bilayer fluidity that accompanies agglutination of chicken erythrocytes may involve a force-generating system responsible for the motion of membrane proteins, namely, one sensitive to microtubule-disrupting drugs. At the concentrations used, the drugs do not alter the hemagglutination titer of Sendai virus.

The effect of the length of time of preincubation with colchicine or vinblastine prior to the addition of Sendai virus to spin-labeled chicken erythrocytes is illustrated in Fig. 4. The difference in $2A'_{zz}$ between the spectrum of agglutinated cells and the spectrum of control cells is plotted as a function of the time of preincubation with drugs prior to the addition of virus. With short preincubation times, from 5 to 20 min, colchicine inhibits the fluidization of the membrane bilayer after virus attachment. However, for longer periods the spectral change that accompanies agglutination is not completely inhibited. The effect of vinblastine is somewhat longer lasting, declining slightly after 60 min of preincubation.

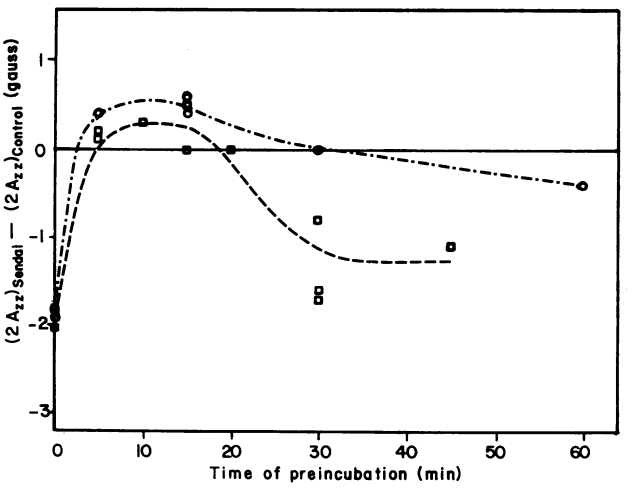

FIG. 4. Inhibition by preincubation with colchicine and vinblastine of the bilayer fluidity increase due to agglutination of chicken erythrocytes with Sendai virus. Difference between splittings of spectra of agglutinated cells and control cells $[(2A'_{zz})_{Sendai} - (2A'_{zz})_{Control}]$ is plotted as a function of the time of preincubation of cells with drugs prior to the addition of virus. Concentrations of cells and virus are defined in Fig. 1. (- - -) Colchicine, 10^{-4} M; (- · -) vinblastine, 10^{-5} M.

DISCUSSION

We have examined the interactions of the enveloped viruses, influenza and Sendai, with erythrocyte membranes as a model system for the early events in virus infection. The envelope of Sendai virus grown in Madin-Darby bovine kidney cells contains one biologically active glycoprotein containing hemagglutinin and neuraminidase activities, and one inactive glycoprotein which can be activated by trypsin treatment to give hemolytic and cell fusion activities (7). Influenza virus possesses one glycoprotein with hemagglutinating activity and another with neuraminidase activity (2). Influenza and parainfluenza viruses adsorb to sialic acid-containing cell receptors through the glycoprotein with hemagglutinating activity. For human erythrocytes, the 55,000-dalton protein termed glycophorin contains about 70% of the membrane-bound sialic acid (20), and has been identified as the receptor for influenza virus as well as for various lectins (4). The chicken erythrocyte has several more sialic acid-containing glycoproteins than the human, although only two of these react with radioactive surface-labeling reagents (21).

Attachment of viruses to the glycoprotein receptor(s) on the chicken erythrocyte surface modifies the interaction of the receptor protein with the lipid bilayer, resulting in an increase in the bilayer fluidity detected by three spin labels. The change in membrane structure accompanying virus attachment is mimicked by the attachment of the lectins, concanavalin A and wheat germ agglutinin. The similarity of the effects of such diverse agents as viruses and lectins is striking, and provides several implicit controls. The fluidity increase after addition of virus is inhibited by pretreating cells with proteases or neuraminidase, demonstrating that attachment to specific glycoprotein receptors is required for the membrane bilayer to become more fluid.

The spectral changes accompanying agglutination of labeled chicken erythrocytes are inhibited by pretreatment with colchicine, vinblastine, or the local anesthetic tetracaine, while cytochalasin B has no effect. The effect of colchicine, and to a lesser extent, that of vinblastine, is dependent upon the length of time of incubation with the drug prior to the addition of virus. Although the nature of this time dependence of colchicine action is not clear, it emphasizes the caution required in interpreting drug sensitivities based upon few time points.

Lateral movement of proteins in the plane of the membrane may account for the lipid fluidity increase accompanying agglutination of chicken erythrocytes. Receptors on the avian erythrocyte have been observed to undergo changes in surface distribution after attachment of multivalent ligands (unpublished data cited in ref. 22). Some forms of membrane protein rearrangement that follow attachment of multivalent ligands appear to be dependent upon structures similar to cytoplasmic microtubules or microfilaments in their drug sensitivity. One such process is "cap" formation, the accumulation of ligand-receptor complexes at one pole of the cell after lectin or antibody attachment to the surface of lymphocytes and certain

other cell types. The role of microfilaments and microtubules in membrane protein mobility is not clear. Microtubule-disrupting drugs such as colchicine may inhibit (18, 23), induce (19, 24), or have no effect (17, 19, 25) on cap formation, depending upon the cell type and ligand involved. Cytochalasin B, which interferes with the function of microfilaments, inhibits cap formation in some systems (17, 19, 25) but not in others (23). Local anesthetics have been observed to inhibit cap formation (26). Thus, the drugs that inhibit the increase in bilayer fluidity upon agglutination of chicken erythrocytes also inhibit certain forms of membrane protein mobility.

Avian erythrocytes contain microtubules arranged in a "marginal band" just beneath the plasma membrane, while human erythrocytes do not have this structure (27). The structural changes in the membrane lipid bilayer seen upon attachment of viruses or lectins to chicken erythrocytes are not observed in analogous experiments with spin-labeled human erythrocytes or with the isolated membranes of either species. The sensitivity to microtubule-disrupting drugs of the membrane structural changes upon agglutination of chicken erythrocytes suggests that the absence of microtubules or related structures in human erythrocytes accounts for the fact that no spectral change occurs upon agglutination of those erythrocytes.

The structural change in the lipid bilayer upon attachment of viruses or lectins to glycoproteins on the chicken erythrocyte surface probably involves several steps. The attachment of virus or lectin to the receptor protein is an event that is spatially separate from the change in the interaction of the receptor protein with the lipid bilayer. Binding of viruses or lectins to receptors is coupled to the structural change in the lipid phase through a drug-sensitive system, possibly involving microtubules on the interior surface of the plasma membrane. The nature of the coupling mechanism is of considerable interest. The lateral redistribution of receptor molecules within the plane of the membrane is a plausible hypothesis, although other forms of plasma membrane activity may be involved. The structural changes in the plasma membrane upon virus attachment, the similarity of these effects to those of lectins, and the possible involvement of microtubule-like systems have interesting implications for the initial events in virus infection.

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1. Choppin, P. W. & Compans, R. W. (1975) in *Comprehensive Virology*, eds. Fraenkel-Conrat, H. & Wagner, R. R. (Plenum Press, New York), Vol. 4, pp. 95–178.
2. Compans, R. W. & Choppin, P. W. (1975) in *Comprehensive Virology*, eds. Fraenkel-Conrat, H. & Wagner, R. R. (Plenum Press, New York), Vol. 4, pp. 179–252.
3. Nicolson, G. L. (1974) *Int. Rev. Cytol.* **39**, 89–190.
4. Tomita, M. & Marchesi, V. T. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2964–2968.
5. Williams, J. C., Melhorn, R. & Keith, A. D. (1971) *Chem. Phys. Lipids* **7**, 207–230.
6. Choppin, P. W. (1969) *Virology* **39**, 130–134.
7. Scheid, A. & Choppin, P. W. (1974) *Virology* **57**, 475–490.
8. Landsberger, F. R., Lenard, J., Paxton, J. & Compans, R. W. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2579–2583.
9. Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130.
10. Blanchet, J. P. (1974) *Exp. Cell Res.* **84**, 159–166.
11. Landsberger, F. R., Paxton, J. & Lenard, J. (1971) *Biochim. Biophys. Acta* **266**, 1–6.
12. Lenard, J., Tsai, D. K., Compans, R. W. & Landsberger F. R. (1976) *Virology* **71**, 389–394.
13. Godici, P. E. & Landsberger, F. R. (1975) *Biochemistry* **14**, 3927–3933.
14. Hubbell, W. L. & McConnell, H. M. (1971) *J. Am. Chem. Soc.* **93**, 314–326.
15. Jost, P., Libertini, L. J., Hebert, V. C. & Griffith, O. H. (1971) *J. Mol. Biol.* **59**, 77–98.
16. Jost, P., Brooks, U. J. & Griffith, O. H. (1973) *J. Mol. Biol.* **76**, 313–318.
17. Taylor, R. B., Duffus, W. P. H., Raff, M. C. & de Petris, S. (1971) *Nature New Biol.* **233**, 225–229.
18. Edidin, M. & Weiss, A. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2456–2459.
19. Edelman, G. M., Yahara, I. & Wang, J. L. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1442–1446.
20. Steck, T. L. (1974) *J. Cell Biol.* **62**, 1–19.
21. Jackson, R. C. (1975) *J. Biol. Chem.* **250**, 617–622.
22. Painter, R. G., Sheetz, M. & Singer, S. J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1359–1363.
23. Ryan, G. B., Borysenko, J. Z. & Karnovsky, M. J. (1974) *J. Cell Biol.* **62**, 351–365.
24. Ukena, T. E., Borysenko, J. Z., Karnovsky, M. J. & Berlin, R. D. (1974) *J. Cell Biol.* **61**, 70–82.
25. de Petris, S. (1974) *Nature* **250**, 54–56.
26. Ryan, G. B., Unanue, E. R. & Karnovsky, M. J. (1974) *Nature* **250**, 56–57.
27. Fawcett, D. W. & Witebsky, F. (1964) *Z. Zellforsch. Mikrosk. Anat.* **62**, 785–806.