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Increase in Lipid Fluidity of Cellular Membranes Induced by Adsorption of RNA and DNA Virions

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Changes in the dynamic behavior of membrane lipids of mammalian cells induced by adsorption of animal viruses were quantitatively monitored by fluorescence polarization analysis with the aid of the fluorescent probe 1,6-diphenyl 1,3,5-hexatriene embedded in the surface membrane lipid core of intact cells. Adsorption of encephalomyocarditis, West Nile, and polyoma viruses to hamster (baby hamster kidney) and mouse (3T3) cells is accompanied by a rapid and significant increase in the degree of fluidity of membrane lipids of the infected cells. These changes in membrane fluidity, which are virus dose dependent, are inhibited by low temperature and by treatment of the cells beforehand with compounds known to block viral receptors on the cell surface. It is suggested that increase in membrane lipid fluidity, induced by the adsorption of virions, is an early event in the process of cell-virus interactions.

Compelling evidence showing that the structural organization of membrane components is of a highly dynamic nature now exists (4, 7, 22). This dynamic behavior of biological membranes is determined, to a large extent, by the degree of mobility of membrane protein receptors (12, 21) and by the degree of fluidity of the membrane lipid bilayer (20, 21). These dynamic features are interrelated since all membrane proteins are embedded, to some extent, in the membrane lipid core (21). It is also a widely accepted fact that the dynamic structural organization of membrane proteins and membrane lipids plays a major role in cellular control mechanisms for cell growth and differentiation (6, 9, 10, 11, 13). During the process of virus-cell interactions, changes in membrane structure and permeability were observed (5, 15). Some of these changes occur a very short time after adsorption of viruses to cells (2; M. Spiegelstein, P. Fuchs, M. Chaimsohn, J. Gitelman, and A. Kohn, J. Cell Biol. 70:414a, 1976).

Recently, fluorescence polarization analysis has been used in the study of dynamic structural properties of membrane lipids in the cell surface of mammalian cells (6, 9–13). The method used is based on fluorescence polarization measurements with the fluorescent probe 1,6-diphenyl 1,3,5-hexatriene (DPH) embedded in the surface lipid bilayer of intact cells (20, 21). This method, shown to be highly sensitive and reproducible, can yield the absolute value of the degree of fluidity in terms of membrane microviscosity (20, 21). In the following study, we have determined the effect of viral adsorption on the degree of membrane lipid fluidity of the infected cells. Our results have demonstrated a rapid increase in the degree of membrane lipid fluidity of baby hamster kidney (BHK) and 3T3 cells after adsorption of encephalomyocarditis (EMC), West Nile (WN), and polyoma (Py) viruses.

MATERIALS AND METHODS

Cells. The mammalian cells used in this study were hamster (BHK-21) and mouse (3T3) cell lines. BHK and 3T3 cells were grown in Falcon tissue culture flasks (250 ml) as monolayers in Eagle medium (Grand Island Biological Co.) containing 10% calf serum. For the experiments, cell monolayers were dissociated with 0.25% trypsin. To avoid the use of trypsin for removing cells from the growing flasks, BHK cells were also grown as suspended cultures in Spinner flasks (16), and the cells were used in the experiments without trypsin treatment. No changes in the degree of membrane lipid fluidity for cells with or without trypsin treatment could be observed.

Viruses. The viruses used in the experiments were EMC, WN, and Py and represented RNA naked picornaviruses, RNA enveloped togavirus, and DNA naked oncogenic papovavirus, respectively. EMC and WN were grown in BHK-21 cells, and Py was grown in 3T3 cells. The concentration and purification of viruses were performed according to methods previously described (14, 24, 25). For the experiments, virus preparations were washed with phosphate-buffered saline (PBS). The infectivity of the viruses was determined as PFU per milliliter. The ratio of PFU to the number of physical particles

has been estimated to be about 10 for EMC virus, between 44 and 47 for Py virus (19), and 90 ± 50 for togaviruses (1). Homogenates of noninfected cells were used as control suspensions for virus preparations. These homogenates were prepared by: (i) thermal inactivation of cells at 60°C for 30 min, followed by Dounce homogenization; (ii) repeated freezing and thawing of cells; or (iii) nitrogen cavitation. Cell homogenate preparations were then centrifuged at 3,000 × *g* for 15 min to remove coarse debris, and the supernatant fluid was used as a control to represent that fraction of the cell content that would appear as contamination in the virus preparations due to similar density. In some experiments the supernatant fluid of the cell homogenate preparations was subjected to a gradient centrifugation, and only the fraction with a density similar to that of the EMC particles was used as control. For UV inactivation of EMC, a 1-ml sample of the virus was irradiated for 30 min in 60-mm petri dishes from a distance of 30 cm by a Westinghouse Sterilamp (G-15-T8) at 870 ergs/s per cm^2. This treatment resulted in a complete loss of viral infectivity (PFU per milliliter) but had no effect on the adsorption kinetics of virus to cells as determined with the aid of [^{3}H]leucine-labeled EMC virus. The ^{3}H-labeled EMC viruses were also used to determine the adsorption of EMC particles to cells at low (4°C) and high (37°C) temperatures.

Fluorescence labeling of cells. The fluorescent probe used in the present experiments was DPH. For labeling of cells, a DPH dispersion was freshly prepared by injection of 0.1 ml of DPH stock solution of 2×10^{-3} M DPH in tetrahydrofuran into 100 ml of vigorously stirred PBS, pH 7.2, at 25°C. BHK-21 and 3T3 cultures grown as monolayers were washed three times with PBS and then incubated for 30 min at 37°C with 5 ml of DPH dispersion, after which 3 ml of 0.25% trypsin was added to each plate (13, 20). The dissociated cells were washed three times with PBS. The DPH-labeled cells were then resuspended in PBS to a concentration of 0.5×10^{-6} to 10^{-6} cells per ml. BHK-21 cells grown in suspension were labeled with DPH under similar conditions but without trypsin treatment. For the inhibition of EMC virus adsorption, DPH-labeled BHK-21 cells were treated for 30 min at 37°C with protamine sulfate at a final concentration of 0.5 ml/mg before addition of EMC virus.

Fluorescence polarization analysis. The degree of membrane lipid fluidity was quantitatively monitored by fluorescence polarization analysis of DPH-labeled cells with the aid of the Elscint microviscosimeter, model MV-1 (Elscint, Haifa, Israel). For excitation a 365-nm band generated from a 200-W mercury arc was passed through a polarizer. The fluorescence light was detected in two independent cross-polarized channels equipped with polarizers after passing through cut-off filters for wavelengths below 390 nm (10, 13). The method used to determine the degree of fluorescence polarization (P) and the degree of membrane microviscosity ($\bar{\eta}$) is comprehensively described in previous articles (9, 10, 13, 20, 21). In principle, high P or $\bar{\eta}$ values represent low lipid fluidity and vice versa. All fluorescence measurements in the present study were carried out at 10 or 37°C. The accuracy of the P values obtained with the Elscint microviscosimeter was ±0.005.

RESULTS

To study possible changes in the degree of fluorescence polarization, P, of DPH-labeled BHK-21 cells as a function of EMC virion adsorption, the P values of the labeled cells were determined before and after addition of the virions. The results showed a constant and significant increase in the degree of membrane lipid fluidity (decrease in P or $\bar{\eta}$ values) of the labeled cells after adsorption of EMC virions (Fig. 1) as compared with untreated BHK-21 cells or cells treated with cell homogenates obtained from noninfected BHK-21 cultures (Fig. 1). This increase in membrane fluidity occurred within 1 to 2 min after addition of the virions and persisted for at least 20 min. A similar increase in membrane lipid fluidity induced by the EMC virions was obtained with BHK-21 cultures grown as monolayers or grown as suspended cultures (Fig. 2). Thus, the trypsin treatment had no effect on the degree of membrane lipid fluidity as determined by fluorescence polarization analysis of DPH-labeled cells (20) or on the effect induced by the EMC virions. The increase in cell membrane fluidity, found to be proportional to the concentration of the virus added, gradually increased from 0.5 to 15 PFU/cell (Fig. 3) and occurred both with infective and noninfective (UV-inactivated) virions (Fig. 4). There was no cell agglutination induced by the virus in this system, and thus the possibility of virus-mediated cross-linking between cells as a cause of changes in the fluidity of membrane lipids can be eliminated. UV inactivation completely inhibited viral infectivity but had no effect on viral adsorption. From these observations, we concluded that the major parameter determining the increase in membrane lipid fluidity is the adsorption of EMC virions to the cell surface membrane. To test this assumption, DPH-labeled BHK-21 cells were treated with protamine sulfate, a procedure known to inhibit part of the receptors for binding of EMC virions on the cell surface (23). The results indicated that the virus effect on the cell membrane fluidity was greatly reduced by treatment of cells with protamine sulfate prior to the addition of the EMC virion (Fig. 5). In the absence of EMC virions, similar P and $\bar{\eta}$ values were obtained with DPH-labeled BHK-21 cells with or without protamine sulfate treatment. Additional evidence for the suggestion that the main effect is associated with the viral adsorption was obtained from experiments showing that changes in the P or $\bar{\eta}$

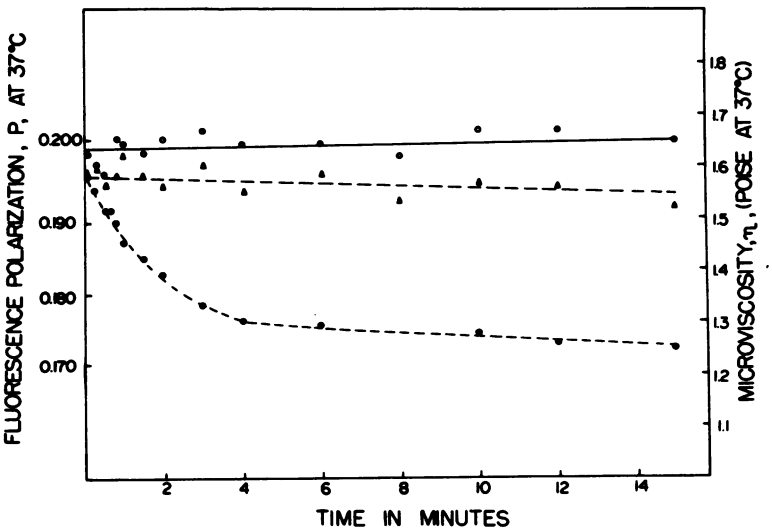

FIG. 1. *Changes in the degree of fluorescence polarization, P, and membrane microviscosity, $\bar{\eta}$, of DPH-labeled BHK-21 cells induced by adsorption of EMC virus. BHK-21 cells, grown as suspended cultures, were labeled with DPH, washed with PBS, and resuspended in PBS to a concentration of 10^6 cells per ml. Samples of 2.7 ml of cell suspension were mixed with 0.3 ml of PBS (○), BHK homogenate (▲), and EMC virus (●). The degree of fluorescence polarization was determined with the aid of the microviscosimeter at 37°C from zero time up to 15 min. The concentration of EMC was 6 PFU/cell. For a control, cell homogenate containing the same amount of protein as the virus preparation was used. Similar results were obtained when a fraction of the cell homogenate with a density similar to that of the EMC particles was used.*

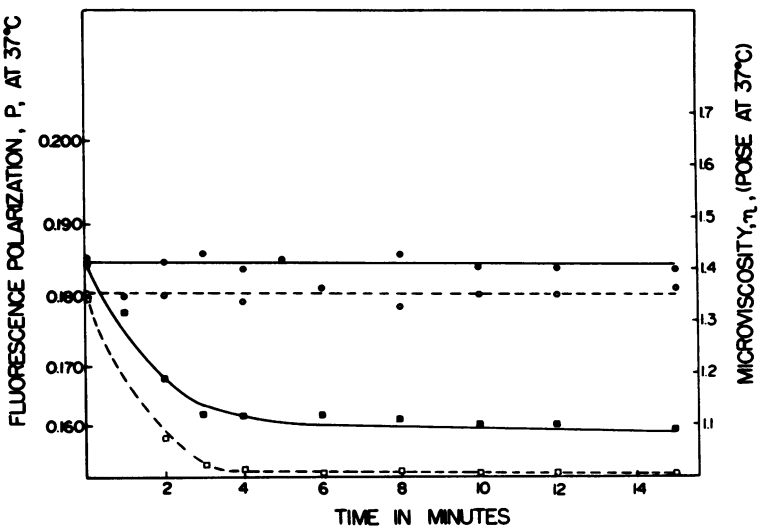

FIG. 2. *Decrease in P and $\bar{\eta}$ values of DPH-labeled BHK-21 cells grown as monolayers and as suspended cultures induced by adsorption of EMC virus. Symbols: suspended BHK-21 cells (●) and after adsorption of EMC (■); BHK-21 cells grown as monolayers (○) and after adsorption of EMC (□). The degree of fluorescence polarization, P, and membrane microviscosity, $\bar{\eta}$, of DPH-labeled cells were determined at 37°C with and without the addition of EMC virus at a concentration of 15 PFU/cell. To samples of 2.7 ml of cell suspension (10^6 cell/ml) 0.3 ml of PBS or 0.3 ml of EMC in PBS was added.*

values induced by EMC virions were completely abolished when the temperature was reduced from 37 to 10°C (Table 1). It is important to note that a similar number of EMC particles were adsorbed at low and high temperatures, as indicated from the binding of ^{3}H-labeled EMC at 4 and 37°C (Table 2). As expected, in the absence of EMC virions the P or $\bar{\eta}$

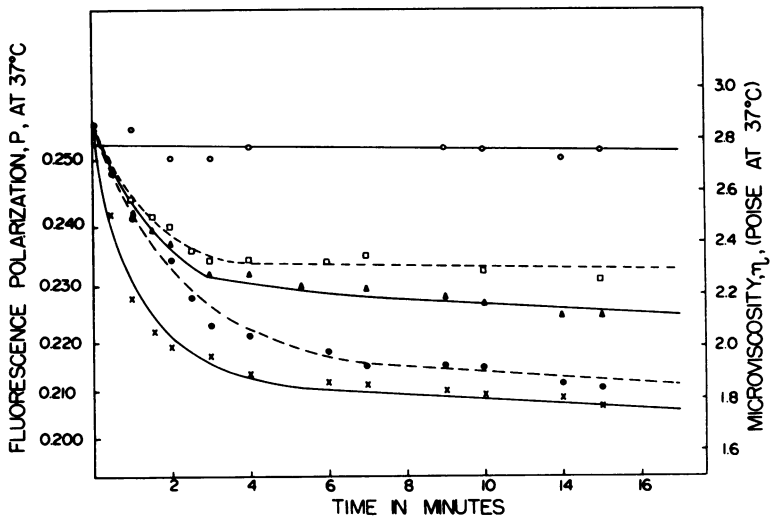

FIG. 3. *Changes in P and $\bar{\eta}$ values of DPH-labeled BHK-21 cells as a function of different concentrations of EMC virus. Samples of 2.7 ml of DPH-labeled BHK-21 cells (suspended cultures) were mixed with 0.3 ml of: PBS* (○); *EMC, 0.5 PFU/cell* (□); *EMC, 1.5 PFU/cell* (△); *EMC, 8 PFU/cell* (×); *and EMC, 15 PFU/cell* (●). *Labeled cell suspensions (0.5 × 10^6 cells per ml) were incubated with viruses, and the degree of fluorescence polarization, P, and membrane microviscosity, $\bar{\eta}$, were determined at 37°C.*

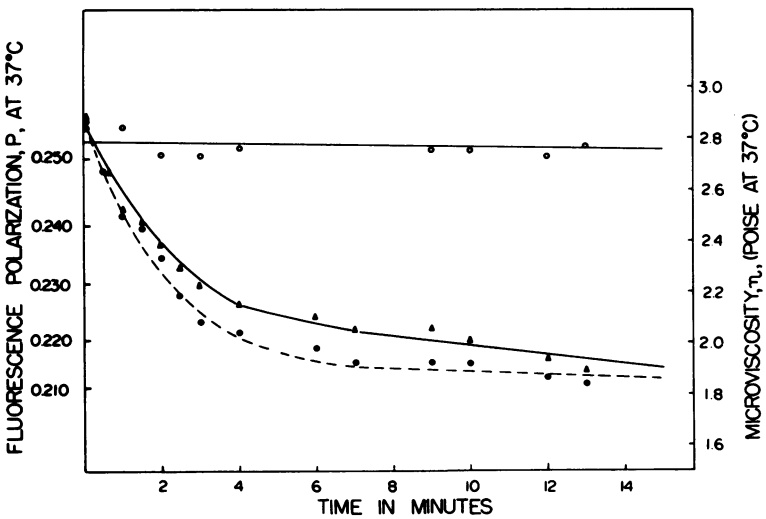

FIG. 4. *Decrease in the degree of fluorescence polarization, P, of DPH-labeled BHK-21 cells and in the membrane microviscosity, $\bar{\eta}$, induced by infective and noninfective EMC virus. Samples of 2.7 ml of BHK-21 cells (10^6 cells per ml) grown as suspended cultures were mixed at 37°C with 0.3 ml of: PBS* (○); *15 PFU of infective EMC virus per cell* (●); *and UV-inactivated EMC virus at the same concentration* (△).

values of the DPH-labeled BHK-21 cells increased (decrease in membrane lipid fluidity) by decreasing the temperature (Table 1). It is evident that the increase in membrane lipid fluidity of DPH-labeled BHK-21 cells induced by the adsorption of EMC virions was not due to cellular components, which might have contaminated the virus preparations since cell homogenates of noninfected cells processed as if they were virus suspensions did not affect the P or $\bar{\eta}$ values of labeled BHK-21 cells (Fig. 1).

All experiments were carried out with DPH-labeled cell suspensions at a concentration of 0.5×10^{-6} to 10^{-6} cells per ml, since under these conditions the P or $\bar{\eta}$ values were independent of cell density (Fig. 6). However, since the P values of cells are markedly affected by the growing conditions and culture age (11),

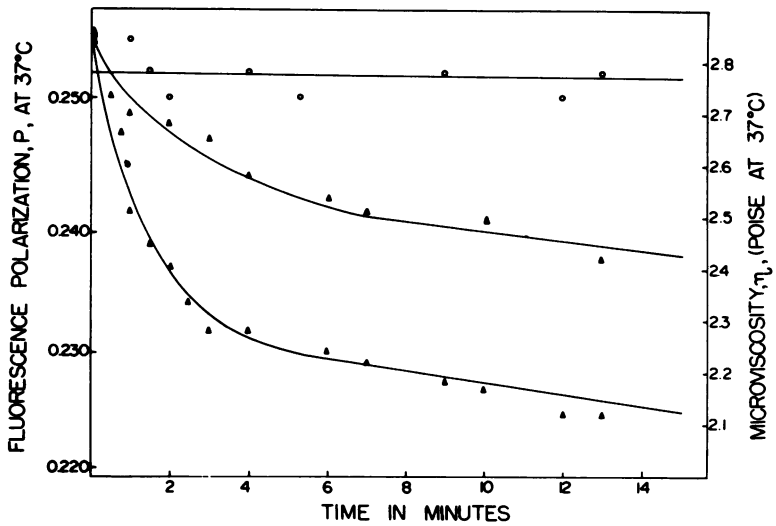

FIG. 5. *Changes in P and $\bar{\eta}$ values of DPH-labeled BHK-21 cells induced by EMC virus with or without treatment of cells with protamine sulfate prior to viral adsorption. Samples of 2.7 ml of DPH-labeled BHK-21 cell grown as suspended cultures with or without protamine sulfate treatment (0.5 mg/ml for 30 min at 37°C) were incubated at 37°C with 0.3 ml of PBS or EMC in PBS as follows: protamine sulfate-treated cells or untreated cells in PBS* (○); *protamine sulfate cells plus EMC at a concentration of 1.5 PFU/cell* (▲); *and untreated cells plus EMC at a concentration of 1.5 PFU/cell* (△).

TABLE 1. *Adsorption of EMC virions to DPH-labeled BHK-21 cells at low and high temperatures*[a]

Time after addition of EMC virions to DPH-labeled BHK-21 cells (min)	Degree of fluorescence polarization (P)	
	Incubation at 10°C	Incubation at 37°C
0	0.296	0.255
2	0.294	0.234
6	0.296	0.218
10	0.294	0.215
16	0.293	0.210

[a] EMC virions at a concentration of 15 PFU/cell were added to DPH-labeled BHK-21 cells at 10 and 37°C. The degree of fluorescence polarization was monitored from zero time up to 16 min after mixing of cells with viruses.

different experiments show different P values. But this variation did not affect the relative changes in the P or $\bar{\eta}$ values induced by the adsorption of EMC virions. The standard conditions for the present experiments were to add 0.3 ml of PBS, cell homogenates, or virus suspension to 2.7 ml of DPH-labeled cells, followed by fluorescence polarization measurements as a function of time from the addition of the virions. It was established that the change in membrane lipid fluidity measured during an experiment was the same whether the DPH-labeled cells were exposed to excitation light only twice at zero time and again after 30 min, or continuously during the 30 min at 2-min intervals.

TABLE 2. *Adsorption of [^{3}H]leucine-labeled EMC virus to BHK-21 cells at low and high temperatures*

Time after adsorption (min)	Adsorption (cpm) at:	
	4°C	37°C
0	6	8
1	69	66
5	392	434
10	634	662
15	712	780
30	952	1,084
60	1,006	1,192
90	1,122	1,218

To test the possibility that the increase in lipid fluidity of cellular membranes is also induced by viruses other than EMC, similar studies were carried out with BHK-21 cells infected with WN virus as an example of RNA enveloped viruses and with BHK-21 and 3T3 cells infected with Py virus as an example of DNA naked viruses. The results indicated that adsorption of both WN and Py viruses to BHK-21 or 3T3 cells was associated with a significant increase in the degree of membrane lipid fluidity, similar to the effect observed with EMC viruses (Table 3).

DISCUSSION

Changes in the degree of lipid fluidity of cellular membranes induced by adsorption of virions were quantitatively determined by

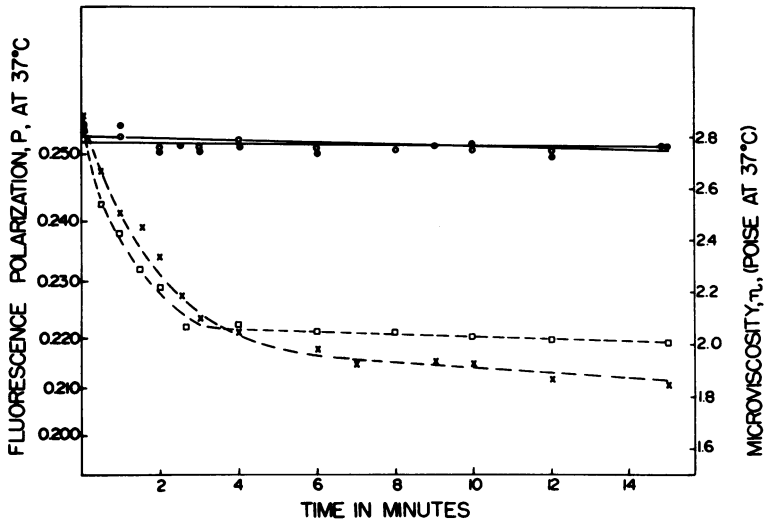

FIG. 6. *Fluorescence polarization, P, and membrane microviscosity, $\bar{\eta}$, of DPH-labeled BHK-21 cells at different cell concentrations with or without adsorption of EMC virus. Symbols: BHK-21 cells (0.5 × 10⁶ cells per ml) without EMC (●) and after adsorption of EMC at a concentration of 15 PFU/cell (×); BHK-21 cells (10⁶ cells per ml) without EMC (○) and after adsorption of EMC at a concentration of 15 PFU/cell (□). All experiments were carried out at 37°C.*

TABLE 3. *Increase in membrane fluidity (decrease in P and $\bar{\eta}$ values) of BHK-21 and 3T3 cells induced by adsorption of EMC, WN, and Py virions*[a]

Time after addition of virions to DPH-labeled cells (min)	EMC/BHK-21		WN/BHK-21		Py/BHK-21		Py/3T3	
	P	$\bar{\eta}$	P	$\bar{\eta}$	P	$\bar{\eta}$	P	$\bar{\eta}$
0	0.185	1.42	0.185	1.42	0.187	1.48	0.221	2.04
2	0.188	1.19	0.159	1.09	0.164	1.15	0.199	1.63
5	0.164	1.15	0.164	1.15	0.158	1.08	1.191	1.51
10	0.160	1.10	0.168	1.19	0.163	1.13	0.187	1.45
15	0.159	1.09	0.165	1.16	0.164	1.15	0.190	1.49
Relative change at 5 min[b]	0.89	0.89	0.89	0.89	0.84	0.73	0.86	0.74

[a] Cell suspensions labeled with DPH were mixed with viruses at 37°C at concentrations of 15 PFU/cell.
[b] Relative change was calculated as the ratio of P and $\bar{\eta}$ values at zero time to P and $\bar{\eta}$ values at 5 min after the addition of the viruses to the cell suspensions.

monitoring the degree of fluorescence polarization, P, of DPH-labeled cells. The present results have clearly indicated that adsorption of EMC and WN viruses to BHK-21 cells and of Py virus to BHK-21 and 3T3 cells is associated with a significant increase in the degree of lipid fluidity of cellular membranes. This increase in the fluidity of membrane lipids, obtained with both infective and noninfective virions, is proportional to the number of virions adsorbed and can be partially inhibited by blocking the viral receptors on the cell surface but is completely inhibited by decreasing the temperature of the interaction from 37 to 10°C. Experiments carried out to determine the adsorption capacity of EMC viruses to cells at low and high temperatures have shown that a similar number of ^{3}H-labeled EMC particles are bound to cells at 4 and 37°C. It can be suggested, therefore, that only at 37°C is the binding of viruses to the cell membrane associated with a rearrangement of membrane proteins, which may be part of the mechanism responsible for the fluidization of the membrane lipid core (3). The experiments also indicated that these changes in the dynamic behavior of membrane lipids can be obtained with both DNA and RNA viruses, as well as with naked or enveloped virions. Three viruses tested in our present study, EMC, WN, and Py, although differing in their structure, composition, and function, produce similar changes in the degree of lipid fluidity of the host cell membranes. It can be concluded, therefore, that increase in lipid fluidity is a general, dynamic structural change of the host membrane associated with early events result-

ing from cell-virus interaction. The major requirement for these changes to occur is the effective adsorption of virions to their receptors on the cell surface membrane.

Recent studies in which attempts have been made to study lipid-lipid interactions in erythrocytes fused with the aid of Sendai virus showed that phospholipid molecules were transferred and intermixed between the lipid phase of the erythrocyte membrane (17). These results, obtained with a virus equipped with a cell-fusing lipoprotein envelope (2, 17), are not surprising because of the intrinisic fusing properties of the viral envelope. It has recently been shown (18) that the microviscosities of togaviruses and of vesicular stomatitis virus labeled with DPH are considerably higher than those of host cell membranes from which these viruses emerge during their maturation. If only an intermixing of the viral envelope with the host cell membrane or a translocation of DPH from the cell membrane to the viral envelope were the cause of the observed changes in the degree of fluorescence polarization, one could, in the case of togavirus, expect a decrease in the degree of lipid fluidity. In our experiments, however, we observed an increase in fluidity with WN virus (Table 3). Thus, even in the case of lipid-containing viruses, the simple lipid intermixing hypothesis is not supported by our findings. Moreover, our results with naked viruses, such as EMC and Py, are of special interest since they demonstrate that changes in the lipid phase of the host cell membrane also occur after the adsorption of virions having no lipid components in their structure.

It has been shown here for EMC virus that even at a multiplicity of 0.5 PFU/cell (equivalent to about five virions per cell [19]), there is a clear fluidization of the host cell membranes by the adsorbed virus. It is clear, therefore, that no mass effect, of the type encountered in fusing enveloped viruses, is involved in this case. The other possibilities to be considered are activation of endogenous, membranal phospholipase after interaction of the adsorbed virus with the host cell and partitioning of the DPH probe into different cellular lipid environments, resulting from pinocytosis of the virions. It has been established that adsorption of viruses to host cells is accompanied by structural and functional changes in the cell surface membrane, such as aggregation of intramembranal particles (2) and changes in the permeability of the membranes (5; Spiegelstein et al., J. Cell Biol. 70:414a, 1976). It will be of interest, therefore, to study to what extent such changes in the structural organization of membrane components are also associated with the fluidization of membrane lipids of the host cells.

Our present experiments have demonstrated that adsorption of virions to cells is accompanied by a marked increase in the fluidity of the host cell membrane lipids. Thus, these observations may serve as a basis for titration of unknown viral suspensions by means of fluorescence polarization analysis. If virus-specific gamma globulins were to prevent the changes in lipid fluidity induced by a virus, whereas nonspecific gamma globulins would not, then perhaps our observations could be developed into a diagnostic test. This possibility is currently under investigation in our laboratory.

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