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# Early Molecular Events in the Interaction of Enveloped Viruses with Cells

## I. A Fluorescence and Radioactivity Study

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**Abstract.** The fluorescence depolarization of 1,6-diphenyl-hexatriene was used to study the dynamic properties of the hydrophobic regions of the lipid envelopes of ortho- and paramyxoviruses as well as of the Rous sarcoma virus and of the membrane lipids of susceptible and nonsusceptible cells.

The systems investigated where active and inactive influenza viruses, and NDV virus acting on chick embryo fibroblasts and Rous sarcoma virus acting on susceptible (C/E) and nonsusceptible (C/B) chicken-cell.

Polarization degrees and mean rotational correlation times of DPH embedded in viral lipids were significantly higher than those of DPH in the cell membranes, due to a higher rigidity of the virus envelopes. When suspensions of labelled viruses and unlabelled cells or unlabelled viruses and labelled cells were mixed, a characteristic change of the fluorescence polarization degrees with time was observed. This behaviour was ascribed to a label transfer from virus to cell membranes or vice versa.

While the rate constants of label transfer from virus to cells and cells to virus were about the same for the penetrating viruses the rate constants of label release from inactive virus to cells were much larger than for the migration in the opposite direction.

**Key words:** Fluorescence polarization degree – Fusion – Label migration – Lipid migration – Cell membrane – Virus envelope.

### Introduction

It is known that molecules belonging to cell membranes like phospholipids (Peterson and Rubin, 1970) and glycolipids (Huang, 1976, 1977) can be translocated between membranes of intact cells. Such a molecular transfer from cell to cell was also observed in the case of the fluorescence label DPH, artificially embedded in the cell membranes and migrating probably by membrane interaction during short cell contact in suspension (Collard et al., 1978).

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It was of interest to investigate whether this label transfer could provide information on virus-cell systems in which virus replication is inhibited at a very early stage of the infection process. The systems analyzed were influenza A virus, Newcastle disease virus (NDV), and Rous sarcoma virus (RSV). With influenza virus, particles can be obtained in which the hemagglutinin glycoprotein of the viral envelope is present either as a larger polypeptide chain or as fragments which are derived from the large form by limited proteolytic cleavage. Similarly, NDV may contain either the cleaved or the uncleaved form of an envelope glycoprotein responsible for cell fusion and hemolysis. In previous studies it has been clearly shown that cleavage is necessary for infectivity of these viruses, but there is only circumstantial evidence that the primary function of these glycoproteins is a direct involvement in the penetration process (Homma and Ohuchi, 1973; Klenk et al., 1975; Lazarowitz and Choppin, 1975; Nagai et al., 1976; Scheid and Choppin, 1974).

In the avian RNA tumor virus system it is well established that the virus susceptibility of chicken embryo cells (CEC) is under genetic control and that the cells obtained from a given embryo have a certain phenotype with respect to their susceptibility to the various virus subgroups (Crittenden et al., 1967). These viruses are also classified according to their host range which in turn is defined by the type of their envelope glycoprotein antigen (Vogt et al., 1967). Therefore, this system allows to compare the interaction of one and the same virus strain with susceptible and resistant cells.

## Materials and Methods

*Viruses.* Strain Italien of NDV, two avian strains of influenza A virus: virus N [A/chick/Germany/49 (Hav2Neq1)] and fowl plague virus [A/FPV/Rostock (Hav1N1)], and the Prague strain, subgroup B, of Rous sarcoma virus (Pr-B virus) have been used.

Seed stocks of the myxoviruses were grown in the allantoic cavity of embryonated eggs and were stored as infected allantoic fluids at −80° C. The Prague strain of RSV has been passaged and cloned in chick embryo cells.

*Virus Growth.* Influenza virus was grown in embryonated eggs and in CEC as described previously (Klenk et al., 1975). NDV was grown in embryonated eggs (Nagai et al., 1976). Virus titres have been determined by hemagglutinin and plaque assays.

The Pr-B virus was grown in CEC cultures from fertilized eggs and stored as tissue culture supernatant at −80° C. The eggs were from a leukosis virus-free flock and kindly supplied by Dr. E. Vielitz, Lohmann-Tierzucht GmbH., Cuxhaven.

*Virus Purification.* Virus was purified from cell culture medium or allantoic fluid by procedures described previously for myxoviruses (Klenk et al., 1972) and for Pr-B virus (Bauer, 1974) respectively.

*Fluorescence Labelling*

1,6-diphenyl-hexatriene (DPH) (Aldrich, puriss) was used as a probe to study the dynamic properties of the lipids in the virus envelopes and in the cell plasma membranes. The virus suspension ($\sim 10^{11}$ particles/ml$^{-1}$) or the cell suspension ($1 \times 10^6$ cells/ml$^{-1}$) in PBS were incubated with the tetrahydrofuran solution of DPH. The molar ratio of DPH : PL[1] was of $\sim 1 : 200$. At this ratio the polarization degree values are constant in time, both for cells and for viruses. The incubation lasted 30 min at 37° C. After incubation, the viruses were washed by centrifuging three times at 100 000 *g* for 20 min and were resuspended in PBS. DPH does not fluoresce in aqueous media.

The cells were washed three times with PBS and finally resuspended in PBS, pH 7.4.

In order to check whether there is any label transfer through the aqueous medium from the labelled to the unlabelled species, DPH-labelled NDV ($\sim 10^{11}$ particles/ml$^{-1}$) were suspended in PBS in a dialysis sack which was immersed in a PBS solution (pH = 7.4) containing $10^6$ cells/ml$^{-1}$. The system was kept for 120 min at 37° C and the fluorescence emission intensity of the cells ($\lambda_{exc} = 365$ nm; $\lambda_{em} = 420$ nm) was measured every 10 min with a Hitachi-Perkin-Elmer MP-2 spectrofluorometer.

**Fluorescence Measurements**

*a) Steady-State Measurements.* Fluorescence polarisation degrees, *p*, were measured with an Elscint microviscosimeter MV-1. The excitation light passed through an interference filter ($\lambda_{exc} = 360$ nm) and a Glan-Thompson polarizer. The fluorescence was filtered by an emission aqueous filter solution of 2 n $NaNO_2$ and analyzed in parallel ($i_\parallel$) and perpendicular ($i_\perp$) directions to the plane of the excitation beam at the same time with two photomultipliers. From the polarisation degree

$$p = \frac{i_\parallel - i_\perp}{i_\parallel + i_\perp}$$

the values for the fluorescence anisotropy were obtained by

$$r = \frac{2p}{3 - p}.$$

The temperature was controlled to $\pm$ 0.1° C using a recording thermoelement and the in-built heating/cooling unit. The fluorescence polarization degree was directly read.

*b) Time-Resolved Measurements.* Fluorescence lifetimes were measured with a single-photon counting instrument, equipped with Ortec electronics, an Ortec multichannel analyser and an Applied Photophysics optical unit with a gated air-filled

[1] PL = phospholipids

flash lamp. Fluorescence decays $d(t)$ and the excitation function $g(T)$, obtained from a light scattering sample, were stored in 256 channels and transferred to a PDP 10 computer. The lifetimes were calculated by fitting a single exponential function

$$F(t) = ae^{-t/\langle\tau\rangle},$$

which is related to the experimental fluorescence decay $d(t)$ by the equation

$$d(t) = \int_0^t F(t - T)\, g(T)\, dT \quad \text{(Wahl, 1975).}$$

$\langle\tau\rangle$ gives an average value for the fluorescence-decay behaviour of DPH in the investigated systems. Test measurements with polarizers rotated at 54° 44′ to each other showed that the influence of depolarization on the value of $\langle\tau\rangle$ could be neglected in all our samples. An exact analysis in terms of fitting a sum of exponential functions which might be pertinent in view of the complex decay behaviour of DPH observed in vesicles of dimyristoyllecithin (DML) (Chen et al., 1977) and egg lecithin (Dale et al., 1977) possibly would provide additional information about the heterogeneity of the dye environment but could not be done for experimental reasons.

For both labelled viruses and labelled cells, successive dilutions in PBS were made and the fluorescence polarization degree measured. The dilution did not affect the polarization degree of the cells, showing no light-scattering depolarization for these systems, whereas in the virus suspension a rather large correction was necessary (Fig. 1). In experiments where labelled viruses were mixed with unlabelled cells or labelled cells with unlabelled viruses, the infection multiplicity dependence of the anisotropy variation with time was studied. Multiplicities of infection from 0.6 pFU/cell to 10 pFU/cell were checked and they were shown to have no influence on the value of the rate constants of either uptake or release of the label. On the other hand, the multiplicity of infection strongly influenced the absolute equilibrium value of the fluorescence polarization.

For the evaluation of transfer rates of DPH-free virus to cells and vice versa we used plots of polarization degrees $p$ vs. time.

In order to characterize the label motion in cell and virus membranes we calculated values for the average rotational correlation time $\langle\varrho\rangle$ from a modified form of Perrin's equation:

$$r = \frac{r_0}{1 + \dfrac{\langle\tau\rangle}{\langle\varrho\rangle}},$$

$r_0$ is the limit anisotropy, when the fluorophores are completely immobilized. $r_0$ was determined to be 0.362 for DPH (Cogan et al., 1973).

This model does not account for static orientations of the probe and $\langle\varrho\rangle$ has to be considered as semiempirical qualitative description of the mobility and, eventually, of orientational constraints of the DPH-molecule in the membrane.

*Radioactivity Measurements*

Influenza virus was grown in chick embryo fibroblasts and purified as described above. The virus was labelled by adding [4-$^{14}$C] cholesterol (Amersham, England, spec. activity 53.7 mg/mmol) and [methyl-$^{3}$H] choline chloride (Amersham, England, spec. activity 10.1 Ci/mmol) at concentrations of 0.2 μCi/mol and 5 μCi/ml, respectively, to the culture medium. Purified virus was suspended in PBS to give final concentrations of about 1000 hemagglutinating units and of $1-2 \times 10^6$ dpm of each isotope per milliliter. 0.5 ml of this virus preparation were added to confluent monolayers of chick embryo fibroblasts on petri dishes (2 cm diameter) that had been washed three times with PBS (37° C). After incubation intervals at 37° C ranging from 5–60 min, the virus was removed from the cells. The monolayers were then rinsed three times with PBS and incubated in 1 ml PBS at 37° C for 30 min with 50 units of neuraminidase from V. cholerae (Boehringerwerke, Germany). The cells were again rinsed three times with PBS, removed from the dishes, pelleted by low speed centrifugation, and resuspended by sonication in 0.5 ml PBS. Aliquots of the cell and virus suspensions were solubilized in toluene (Packard Instrument Company) and radioactivity was analyzed by liquid scintillation counting.

## Results and Discussion

*a. Fluorescence Measurements of DPH in the Virus-Envelope*

The fluorescence polarization degrees, average lifetimes of the excited states of DPH embedded in the virus and the cell lipids and the average rotational correlation times of DPH are listed in Table 1.

The envelopes of ortho- and paramyxoviruses and of Rous sarcoma virus are significantly more rigid than the host cell membranes, in agreement with observations made on the vesicular stomatitis virus (VSV) (Barenholz et al., 1976) and on togaviruses (Moore et al., 1976). This may be due to a restriction of label motion by the following factors:

(i) the degree of saturation of fatty acids of virus envelopes is higher than of the cell plasma membranes,

(ii) the cholesterol : phospholipid molar ratio in the viral envelopes is higher than in the plasma membranes of the host cells.

In the virus envelopes of ortho- and paramyxoviruses, for example, this molar ratio is close to 1 (Klenk et al., 1972; Klenk and Choppin, 1969, 1970) while in the CEC plasma membranes it is $0.27 \pm 0.003$ (Johnson and Robinson, 1978, personal communication).

(iii) a different protein composition of virus and cell membranes.

The difference in the $\langle \varrho \rangle$ values of the influenza virus and of the Rous sarcoma virus, both viruses grown in chick embryo cells, is quite large suggesting that lipids alone, cholesterol : phospholipid ratio included, are not sufficient to account for these differences. The number and types of proteins (glycoproteins) found on the outer face of the lipid bilayer of the Rous sarcoma virus is quite different from that found in the outer face of the lipid bilayer of the influenza virus.

**Table 1.** The fluorescence polarization degree, $p$, the lifetime of the excited state of DPH, $\langle\tau\rangle$, and the average rotational correlation time $\langle\varrho\rangle$ of DPH embedded in the virus envelopes and cell membranes

| System | Fluorescence polarization degree, $p$ 37°C | Lifetime of the excited state $\langle\tau\rangle$, NS and average rotational correlation time $\langle\varrho\rangle$ of 37° C | |
|---|---|---|---|
| | | $\langle\tau\rangle$, ns | $\langle\varrho\rangle$, ns |
| Chick embryo fibroblasts | 0.163 ± 0.005 | 7.6 ± 0.6 | 3.52 ± 0.6 |
| Rous sarcoma virus PrB | 0.337 ± 0.005 | 9.8 ± 0.6 | 16.2 ± 0.6 |
| Trypsinized influenza virus ($N + T$) | 0.261 ± 0.005 | 9.0 ± 0.6 | 9.9 ± 0.6 |
| Nontrypsinized influenza virus | 0.257 ± 0.005 | 9.0 ± 0.6 | 9.6 ± 0.6 |
| Egg-grown influenza virus | 0.307 ± 0.005 | 9.2 ± 0.6 | 15.6 ± 0.6 |
| Newcastle disease virus (egg grown) | 0.298 ± 0.005 | 9.1 ± 0.6 | 14.1 ± 0.6 |
| MDBK cells | 0.142 ± 0.005 | 7.5 ± 0.6 | 3.25 ± 0.6 |

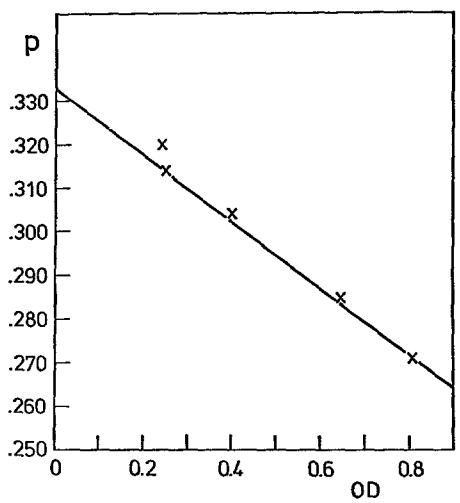


**Fig. 1.** Dependence of the fluorescence polarization degree of DPH in the RSV upon the turbidity of the suspension

For the interpretation of fluorescence investigations in togavirus (Moore et al., 1976) and of spin label results on influenza virus (Landsberger and Compans, 1976) and Sindbis virus (Sefton and Gaffney, 1974) protein-lipid interactions also play an important role. On the other side Barenholz et al. (1976) reported that, for the vesicular stomatitis virus no difference is observed between the fluorescence polarization degrees of lipid extracts and intact virus, but no lifetimes of DPH in this viruses were measured and no calculation of the label mobility is possible.

So it seems, that the role of proteins in influencing the fluidity of the virus lipid bilayer cannot, at the present stage be generalized, at least before reliable fluorescence measurement ($\langle\varrho\rangle$ and $\langle\tau\rangle$) are available.

The fact that cleavage of the spikes by trypsin treatment does not affect either the values of $\langle \varrho \rangle$ or that of average lifetime of the DPH excited state – [similar observations were reported by Landsberger et al. (1971) in a spin label study of the influenz virus] – may be explained by the fact that the proteolytic digestion does not affect any potential fluidity-determining interaction between lipids and proteins.

An important aspect of these type of experiment is the absolutely necessary correction for light-scattering depolarization, which in these systems is quite dramatic. The fluorescence polarization degree of DPH in the cells is little affected by the optical density of the cell suspension at a nonabsorbing wavelength ($\lambda = 450$ nm) (Johnson and Nicolau, 1977) whereas the polarization degree of DPH in viruses is very strongly affected by the suspension turbidity (Fig. 1). The values which we used throughout this paper are those extrapolated for $OD = 0$ in the case of the viruses. Similarly very strong depolarizations were observed with lipid vesicle suspensions (Roche et al., 1978) and with membrane vesicles (Johnson and Nicolau, 1977).

### *b. Virus-Cell Early Interactions*

Influenza virus with cleaved hemagglutinin, i.e., virus *N* grown in eggs, CEC grown virus *N* treated with trypsin, and fowl plague virus, are fully infectious, whereas influenza virus with uncleaved hemagglutinin, i.e., virus *N* grown in CEC, has a reduced infectivity (Klenk et al., 1975). Similarly, strain Italien of NDV contains also cleaved glycoproteins and is also fully infectious (Nagai et al., 1976). The Rous sarcoma virus, Pr-B was used with two kinds of CEC namely of phenotype C/E, which is susceptible for infection with this virus and of phenotype C/B line, which is not. They differ in so far as the latter does not have the receptors for the RSV Pr-B.

When labelled influenza viruses, containing cleaved or uncleaved hemagglutinin, are added to nonlabelled CEC and the fluorescence polarization degree is followed in this system during the first 45 min, a strong decrease of the polarization degree is

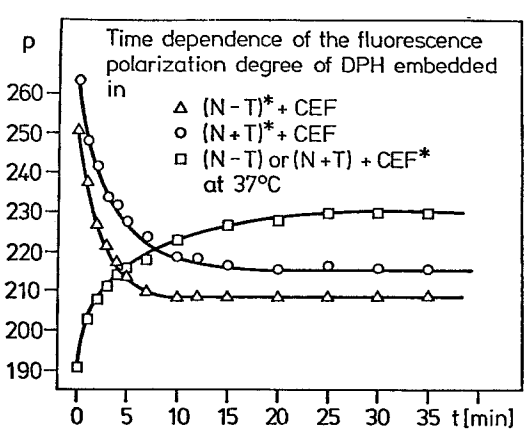


**Fig. 2.** Variation with time of the fluorescence polarization degree of DPH-labelled influenza virus cell systems. The asterisk indicates the labelled entities, $t_0 = 37°$ C in PBS, pH = 7.4

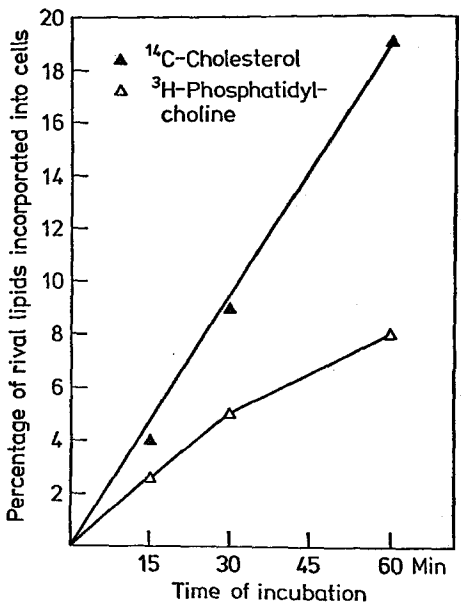


**Fig. 6.** Transfer of viral lipids into cells. Chick embryo fibroblasts were stimulated with fowl plaque virions contains radioactivity labelled cholesterol and phospholipids, and the amount of lipid label incorporated into cells has been determined as described in the text. ▲: [$^{14}$C]-cholesterol; △: [$^{3}$H]-cholin-labelled phospholipids

b) It was of interest to find out whether lipid transfer occurs from the virus into cellular membranes resulting in an alteration of the lipid composition of these membranes. Chick embryo cells were exposed to fowl plague virions containing $^{14}$C-labelled cholesterol and $^{3}$H-choline labelled phospholipid and the kincetics of the incorporation of both isotopes into the cells has been determined. The incorporation rate of cholesterol was significantly higher than that of the phospholipid indicating that there was a preferential transfer of cholesterol from the virus to the cell (Fig. 6). Similar results have been obtained when virus *N* in both the active and the inactive form has been used. The observed half-lifetimes in our fluorescence experiments were in the range of 1–3 min, that means much faster than the processes a) and b). From this fact we conclude that we observe mainly the process c). Treatment of DPH-labelled cells with neuraminidase was without influence on the fluorescence polarization degree. Recently Moore et al. (1978) observed that interaction of the vesicular stomatitis virus with excess unilamellar phosphatidylcholine vesicles resulted in depletion of 90% of the cholesterol from the membrane of intact virus. This depletion caused a substantial increase in the fluidity of the virus membranes as measured by fluorescence depolarization of DPH. The interaction of the virus with the vesicles resulted also in a significant loss of infectivity of the virus. The half-lifetime of cholesterol depletion was of about 6 h (Moore et al., 1978).

As Moore et al. (1978) point out the presence of residual adsorbed vesicles contributed significantly to the increased fluidity measured by fluorescence depolarization in the cholesterol depleted virus membranes. This observation supports findings concerning the decrease in the fluorescence anisotropy of DPH observed after adsorption of the labelled viruses into the unlabelled cells.

*Kinetic Analysis*

In an attempt to differentiate between penetrating and nonpenetrating viruses, based upon the fluorescence date, a kinetic analysis of the time course of the fluorescence polarization degree with time was made.

**Table 2.** Rate constants and half-lifetimes of the DPH transfer in the system envelopes viruses-chick embryo fibroblasts, at 37° C

| System | $k_1$, $min^{-1}$ ± SD | $t_{1/2}$, min ± SD | Confidence limit ($p > 0.99$) |
|---|---|---|---|
| CEF + $(N-T)$* | 0.410 ± 0.012 | 1.69 ± 0.05 | 1.53–1.85 |
| CEF* + $(N-T)$ | 0.233 ± 0.020 | 2.97 ± 0.26 | 2.19–3.75 |
| CEF + $(N+T)$* | 0.297 ± 0.014 | 2.33 ± 0.11 | 1.98–2.67 |
| CEF* + $(N+T)$ | 0.237 ± 0.019 | 2.92 ± 0.23 | 2.23–3.61 |
| CEF + $N_{egg}$* | 0.330 ± 0.033 | 2.10 ± 0.21 | 1.50–2.70 |
| CEF* + $N_{egg}$ | 0.369 ± 0.016 | 1.88 ± 0.08 | 1.65–2.11 |
| CEF + NDV* | 0.215 ± 0.010 | 3.22 ± 0.15 | 2.75–3.69 |
| CEF* + NDV | 0.212 ± 0.015 | 3.27 ± 0.23 | 2.58–3.69 |
| C/B + RSV* | 0.210 ± 0.008 | 3.30 ± 0.13 | 2.94–3.66 |
| C/B* + RSV | 0.117 ± 0.006 | 5.92 ± 0.30 | 5.07–6.77 |
| C/E + RSV* | 0.200 ± 0.008 | 3.47 ± 0.14 | 3.05–3.87 |
| C/E* + RSV | 0.251 ± 0.013 | 2.76 ± 0.14 | 2.34–3.18 |

* DPH labelled

Using a nonlinear least-squares method the time dependence of the fluorescence polarization degree in the cell-virus systems was fitted by a mono-exponential function. The use of a biexponential fit did not improve the agreement with the experimental data beyond the standard deviation. The results are listed in Table 2. Examination of the rate constants and half-lifetimes in Table 2 shows:

*a) Myxo-(paramyxoviruses):*

1) The labelled cells show the same half-lifetimes for the increase in their fluorescence polarization degrees with both infectious $(N + T)$ and noninfectious influenza virus $(N - T)$.

2) The half-lifetimes of label transfer are very close both for the labelled cells and the labelled virus for the $(N + T)$-CEC systems and identical for the NDV-CEC systems.

3) In the case of the noninfectious, nonpenetrating $(N - T)$ influenza virus, the label release by the viruses is significantly faster than the release by the cells

($t_{1/2}$ virus = 1.69 min and $t_{1/2}$ cell = 2.97 min).

*b)* In the case of the Rous sarcoma virus, Pr-B the $t_{1/2}$ values for the DPH release by the virus and by the cells are quite close (Table 2) while for the nonsuscep-

tible cells these values are different ($t_{1/2}$ virus $\approx 2\ t_{1/2}$ cell). Here the main difference is due to changes in $t_{1/2}$ of label release from the cells while the values for label release from the virus are the same for the two cell lines.

In the mixtures of nonpenetrating viruses and cells the label transfer from virus to cell is much faster than in the opposite direction while in mixtures of penetrating viruses and cells the DPH transfer rate is about the same in both directions.

These results could be explained by a complex kinetic model (Perram et al., 1978) (following paper).

It must be mentioned that DPH follows the distribution of the phospholipids in cell membranes (Johnson and Nicolau, 1977). It has been shown, with different cells, that, at 25° C the half-lifetime for DPH incorporation in the cell membranes is 1.5 min (Moore et al., 1976), well below our values, obtained at 37° C. The values of $p$ which we measure are average values for all the cell membranes, and represent therefore, in the virus-cell systems equilibrium values.

Recently Levanon and Kohn (1978) investigated the changes in lipid fluidity of cellular membranes induced by adsorption of RNA and DNA virions.

They reported that paramyxoviruses induce an increase in fluorescence polarization of the cell membranes whereas orthomyxoviruses have the opposite effect. These findings were explained by a fusion mechanism of penetration for the paramyxoviruses and a different penetration mechanism for the "fluidizing" viruses.

As no correction for light-scattering depolarization upon the virus addition to the labelled cells and no lifetime measurements were made by these authors, we believe that these experiments have to be checked regarding the controls (scattering depolarization corrections above all) before reasons for the discrepancies between their results and ours should be sought.

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