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Biochimica et Biophysica Acta 1190 (1994) 360–366

BBA Biochimica et Biophysica Acta

On the validity of lipid dequenching assays for estimating virus fusion kinetics

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(Received 5 October 1993)

Abstract

Octadecylrhodamine (R18) has often been used to measure membrane fusion of enveloped viruses by fluorescence dequenching. In order to see whether non-specific R18 exchange between non-fused membranes occurs we have measured fusion of influenza virus with erythrocyte membranes by utilizing dequenching of the non-exchangeable lipid analogue *N*-(lissamine-rhodamine B-sulfonyl)diacylphosphatidylethanolamine (N-Rh-PE). Rather low concentration of N-Rh-PE (< 0.1 mol%) were required to assess fusion since self-quenching in the influenza virus membrane was more efficient in comparison to R18. For both markers we observed the same kinetics as well as the same extent of fluorescence dequenching upon triggering low pH-induced fusion. Non-specific marker transfer was not observed. Haemolysis was not affected by either type of fluorophore. Our results confirm that R18 is a valuable tool to investigate membrane fusion of enveloped viruses in a quantitative manner. Differences in the efficiency of self-quenching of both markers are discussed.

Key words: Influenza virus; Fusion; Erythrocyte; Fluorescence; Rhodamine

1. Introduction

In recent years, many studies on the kinetics of lipid mixing as a result of fusion between enveloped viruses and target membranes have been made to elucidate the spatial and temporal structure of the fusion site. Fluorescence assays have become a valuable tool for those kinetic studies monitoring continuously fusion by intermembrane mixing of incorporated fluorophores. Assays are based on intermolecular interactions between fluorophores which are sensitive to their membrane concentration, e.g., resonance energy transfer between different fluorophores and self-quenching of fluorophores, respectively. Lipid-like fluorophores with one fatty acyl chain have been introduced which can be incorporated easily and efficiently into biological membranes. For example, Hoekstra et al. [1] have developed a fluorescence dequenching assay to monitor viral fusion with their target membrane, based on the relief of self-quenching upon fusion of the 'lipid-like' probe octadecylrhodamine (R18) initially incorporated into virus membranes. This assay has found widespread application in the field of membrane fusion.

When using these assays it is necessary to account for (i) any fluorophore associated modification of viral fusogenic activity and (ii) non-specific label exchange between closely apposed, but not fused membranes. These points have been raised for the R18 assay very recently [3,4]. The authors have found a gradual inactivation of influenza virus A/PR 8/34 with increasing concentration of R18. Moreover, a significant nonspecific transfer of R18 from labelled viruses to small unilamellar vesicles [3] and – although to a lesser extent – to large unilamellar vesicles (LUV) was observed [4]. However, Hoekstra et al. [1] have pointed out, that R18, once intercalated into the membrane, did not dissociate either from artificial membranes (LUV) nor from biological membranes (Sendai virus, erythrocytes).

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Abbreviations: HA, hemagglutinin; PBS, phosphate-buffered saline; R18, octadecylrhodamine B chloride; N-Rh-PE, *N*-(lissamine-rhodamine B-sulfonyl)diacylphosphatyldiethanolamine; FDQ, fluorescence dequenching; RBC, red blood cell.

SSDI 0005-2736(93)E0366-I

Since the R18 assay has become a prevalent approach to study virus fusion a careful investigation of possible pitfalls is required. Utilization of different fluorophores in membrane-mixing assays should be helpful in clarifying of these issues. Non-specific marker exchange may be circumvented by bio-synthetic labeling of viral lipids [5,6] or by incorporation of fluorescent lipid probes having two long fatty acid chains. In the present paper we have also employed such a probe, the lipid analogue *N*-(lissamine-rhodamine B-sulfonyl)-diacylphosphatdiylethanolamine) (N-Rh-PE), to label influenza virus and to assess low pH-induced fusion of influenza virus with the erythrocyte membranes using the same virus as Wunderli-Allenspach and Ott [4]. It has been shown that N-Rh-PE is a non-exchangeable lipid analogue when incorporated into liposomes or cell membranes [7–9]. We found that self-quenching of the fluorophore was very efficient in the virus membrane. Thus, rather low concentrations of the N-Rh-PE were required for monitoring fusion between influenza virus A/PR 8/34 and erythrocyte membranes. We have compared the fusion kinetics and extent measured with N-Rh-PE with those obtained using the R18. The plasma membrane of human erythrocyte bearing sialic acid receptors has been widely used as model target membrane to investigate low pH-triggered fusion of influenza virus. The fusion process is regulated by the pH of the suspension medium [10,11]. The particular advantage of using acid-triggered viral fusion models in these studies is that the fusion event is dominated by virus bound to the membrane at the time of mixing. Both binding to receptors of the plasma membrane and fusion with the target membrane have been associated with the viral spike protein, the hemagglutinin (HA) [12,13].

2. Materials and methods

Materials. Octadecylrhodamine B chloride (R18) was purchased from Molecular Probes (Junction City, OR), N-Rh-PE was from Avanti Polar Lipids, Inc. (Alabaster, AL). Fresh blood from healthy donors was obtained from the Blood Bank, Berlin-Lichtenberg, and was used within 3 days after sampling (ACD storage medium). Purified influenza virus was kindly provided by Dr. A Lesnau from the Bundesgesundheitsamt (Berlin-Lichtenberg).

Red blood cell and ghost preparation. After removal of buffy coat and plasma red blood cells (RBC) were washed three times in phosphate-buffered saline (PBS, pH 7.4). Unsealed erythrocytes were prepared according to Dodge et al. [13].

Labelling of virus for fusion. 2 μl of a 2 mM stock solution of R18 in ethanol were added under rapid vortexing to 0.5 ml of A/PR 8/34 (1 mg virus protein/ml) [15;16]. Labelling of virus membranes with N-Rh-PE was most efficient when 6 μl of a 1 mM stock solution in chloroform were mixed with 0.6 ml PBS. Subsequently, 0.5 ml of virus suspension were added under vortexing at room temperature. After incubation for 30 min at room temperature (in the dark) viruses were washed five times with ice-cold PBS to remove unbound fluorophore, and resuspended to a concentration of 1 mg/ml. Protein concentration of ghosts as well as of viruses was determined according to Lowry. The amount of fluorophore incorporated into the virus membrane was obtained by measuring the fluorescence intensity in the presence of 0.5% Triton X-100. The membrane concentration of the fluorophore was estimated by assuming that 1 mg of virus protein corresponds to 0.3 mg virus lipid [17].

Virus binding to cell membranes. 0.1 mg of labelled A/PR 8/34 was incubated for 45 min on ice with 0.2 ml of erythrocyte ghost suspension (6–7 mg ghost protein/ml). (In some cases, A/PR/ 8/34 was inactivated by preincubation at low pH (5.0), 37°C, for 10 min in the absence of target membranes.) The suspension was then washed in 10–15 vol. of ice-cold PBS and resuspended corresponding to a concentration of 1 mg virus/ml.

Fusion analysis. Fluorescence dequenching of labelled virus attached to ghost membranes was measured using a SHIMADZU PC5001 spectrofluorometer. Usually, fluorescence traces were recorded with a 0.5 s time resolution. 30 μl of virus-RBC suspension was transferred in a standard cuvette containing 2 ml of prewarmed PBS (pH 7.4). The suspension was stirred continuously with a 2×8 mm Teflon-coated magnetic stirring bar. Low pH-induced fusion of influenza virus was triggered by injecting appropriate amounts of 0.25 M citric acid.

The fluorescence was monitored for 480 s ($\lambda_{ex} = 560$ nm, $\lambda_{em} = 590$ nm, cut-off filter 570 nm, slit width $\Delta\lambda_{ex} = 5$ nm, $\Delta\lambda_{em} = 10$ nm), after which Triton X-100 (0.5% final concentration) was added to obtain maximum fluorescence $F(\infty)$. The percentage of fluorescence dequenching FDQ was calculated as described previously [18]:

$$\% \text{ FDQ} = 100 \cdot [F(t) - F(0)]/[F(\infty) - F(0)] \quad (1)$$

with $F(0)$ and $F(t)$ corresponding to the fluorescence intensity of the virus before starting fusion and the fluorescence intensity at a given time t, respectively.

Accessibility of fluorophores. To assess the transbilayer orientation of lipid like fluorophores (R18, N-Rh-PE) fluorescence intensity was measured in the presence of the aqueous non-permeable quencher KJ. Accessibility of the fluorophores to KJ was analyzed by a modified Stern-Volmer equation according to Lehrer [19]. When a fluorophore has two binding sites one of

which is inaccessible to the quencher the Stern-Volmer equation can be written as:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{f_a K[Q]} \quad (2)$$

where F_0 and F are the fluorescence intensities in the abscence and presence of KJ, respectively, f_a is the fraction of fluorophore accessible to KJ, K is the quenching constant and [Q] is the concentration of the quencher. If all fluorophores are accessible to KJ, f_a becomes 1.

Haemolysis assay. 40 μg of virus were bound to $2 \cdot 10^8$ intact red blood cells in 40 μl of buffered saline (pH 4.6–6.0, 0°C). After 5 min (0°C) 1.8 ml of prewarmed buffer (37°C; pH 4.8–6.0) was added. Following an incubation at 37°C for 5 min the suspension was centrifuged and the supernatant (0.4 ml) was diluted in 2 ml of 0.5% NH_4OH (+50 μl 20% Triton). The extinction was measured at 540 nm. The reference (100% haemolysis) was determined by mixing of 0.4 ml of the suspension with 2 ml of 0.5% NH_4OH (+50 μl 20% Triton).

3. Results

Labelling of virus membrane with the rhodamine probes R18 and N-Rh-PE

The uptake of N-Rh-PE into the virus membrane is comparable to that of R18. Up to a fluorophore amount corresponding to 4 mol% of endogenous lipids about 60–70% (N-Rh-PE) and 70–90% (R18), respectively, were intercalated into the virus membrane. The emission spectrum of N-Rh-PE incorporated into the lipid phase of influenza virus A/PR 8/34 was compared with those of the fluorophore solubilized in ethanol and chloroform, respectively. In all cases excitation was maximal at 560 nm. The emission spectrum was dependent on the probe environment. The decrease of the solvent polarity was accompanied with an increase of the fluorescence intensity. The wavelengths of maximum emission for both fluorophores were in the order: membrane > ethanol > chloroform (data not shown). Emission spectra of N-Rh-PE were almost identical to that of R18 [1]. Therefore, we can conclude that both fluorophores are located in a similar, rather hydrophilic environment of the virus membrane [1].

Similar to R18 we observed a considerable enhancement of self-quenching of fluorescence with increasing concentrations of N-Rh-PE in the influenza virus membrane. However, self-quenching of N-Rh-PE was significantly higher compared to R18, in particular, at low fluorophore concentration (Fig. 1). A 90% self-quenching of N-Rh-PE was observed at a surface density as low as 0.02 mol% of endogenous lipids. For R18 we

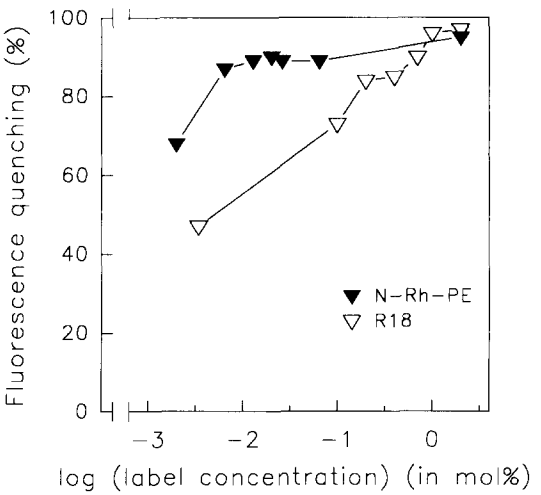

Fig. 1. Self-quenching of R18 and N-Rh-PE, respectively, in the membrane of influenza virus A/PR 8/34 in dependence of their membrane concentration (given in mol% of endogenous virus lipids). Fluorescence dequenching was measured as described in Materials and methods.

observed such a pronounced quenching only at a concentration of ≥ 0.8 mol%. For both probes, quenching was independent of pH.

The fluorescence quenching can be caused either by collisional interaction during the life time of the excited state (dynamic quenching) or by aggregation (static quenching) of the fluorophores. We found a decrease of fluorescence quenching of R18 and N-Rh-PE, inserted into the virus membrane, upon elevating the temperature which is indicative of static quenching. The residual fluorescence intensity of the fluorophores (1 mol%) increased more than two times when shifting the temperature from 4°C to 37°C. This suggests that self-quenching might be caused by aggregates of fluorophores within the membrane that are destabilized by increasing temperature. Fluorescence intensity as a function of temperature gave a straight line in an Arrhenius plot (data not shown).

R18 as well as N-Rh-PE incorporated into the virus membrane are accessible to the aqueous quencher KJ (Fig. 4A), which is known not to permeate biological membranes. The fluorescence decrease in the presence of KJ confirms that fluorophore moieties are located in a rather hydrophilic environment. However, the fluorescence decrease of N-Rh-PE inserted into the virus membrane has a much steeper dependence on concentration of KJ as apposed to R18.

This cannot be explained by a stronger interaction and/or a more efficient quenching between KJ and N-Rh-PE because no difference in quenching by KJ was found for R18 and N-Rh-PE when intercalated into membranes of egg-PC liposomes (data not shown). Formation of liposomes was performed in the presence of fluorophores to ensure a symmetric transversal dis-

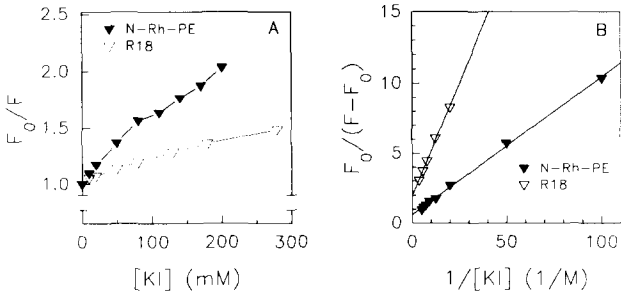

Fig. 2. Stern-Volmer plot (A) and modified Stern-Volmer plot (B) of residual fluorescence of R18 and N-Rh-PE in virus membranes in the presence of the aqueous quencher KJ at 37°C, pH 7.4. F_0 and F are the fluorescence intensities in the absence and presence of KJ, respectively. Lines in (B) were obtained by linear regression. Membrane surface density of R18 and N-Rh-PE corresponds to 1 mol% and 1.1 mol%.

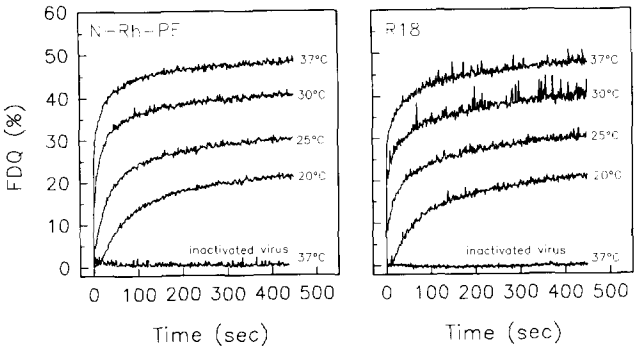

Fig. 3. Kinetics of fusion of labelled influenza virus with erythrocyte membranes at pH 5.0 at different temperatures. A/PR 8/34 was labelled either with R18 or N-Rh-PE. Subsequent to binding, virus-ghost complexes were injected into 2 ml prewarmed buffer of the given pH. Labelling of virus and measurement of fluorescence were done as described in Materials and methods. Time resolution of kinetics is 0.5 s. Influenza virus was inactivated by preincubation at low pH, 37°C, for 10 min.

tribution between both halves of the liposome membrane *.

Another reason for the less efficient quenching of R18 may be that only a fraction of this fluorophore is accessible to KJ. In order to test this notion we have analyzed the KJ-dependent quenching by a modified Stern-Volmer-plot (Fig. 2B) according to Lehrer [19] (see Materials and methods, Eq. (2)). Fitting the data by linear regression we obtained for R18 $f_a = 0.51$ suggesting that only 50% of the fluorophore in the virus membrane is accessible to KJ. In contrast to that, the analysis of the quenching data of N-Rh-PE revealed that all fluorophores are accessible to KJ. It is interesting to note that the quenching constant K obtained by regression is similar for both fluorophores ($K_{R18} = 6.27\ M^{-1}$; $K_{N\text{-}Rh\text{-}PE} = 6.18\ M^{-1}$).

Fusion of labelled influenza virus with erythrocyte membranes

We have compared the kinetics of fluorescence dequenching of R18 labelled viruses with that of N-Rh-PE (Figs. 3 and 4) upon triggering membrane fusion at low pH. As can be seen the time dependence and the extent of fluorescence dequenching was very similar for both fluorophores used. Likewise, the pH and temperature dependence of virus fusion assessed by different labelling procedures was identical (Fig. 4).

No dequenching of virus-ghost complexes was observed at neutral pH suggesting that non-specific exchange of both labels does not occur. Moreover, no fluorescence increase was established at low pH when labelled virus was inactivated by preincubation at low pH, 37°C for 10 min (see Materials and methods) before binding to the target membrane. Likewise, no dequenching was observed when labelled viruses were incubated with nonlabelled viruses. This confirms that the FDQ upon acidification of the suspension medium is indeed caused by fusion of viruses with ghost membranes. The extent of dequenching upon fusion was independent of the amount of marker associated with the virus membrane in the concentration range investigated (not shown).

With intact influenza virus, time delays between triggering and the onset of fusion in the range of 1–10 s have been observed at temperatures below 30°C [11] (see also [20]). In agreement with these results we

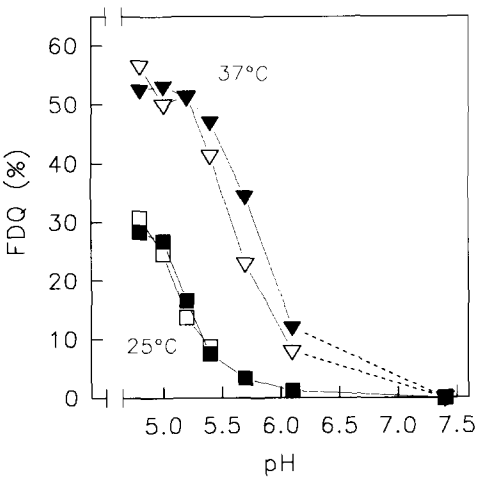

Fig. 4. Fluorescence dequenching caused by low-pH induced fusion of labelled influenza virus with erythrocyte membranes as a function of pH at various temperatures (triangle, 37°C; square, 25°C). Influenza virus was labelled either with R18 (open symbols) or with N-Rh-PE (filled symbols). FDQ was measured as described in Materials and methods.

* Selfquenching of either R18 and N-Rh-PE was less efficient in egg-PC liposomes compared to influenza virus. This is in agreement with results of Hoekstra et al. [1] and Aroeti and Henis [2] who found that the degree of fluorescence dequenching of R18 in Sendai virus membranes was significantly higher than in PC liposomes.

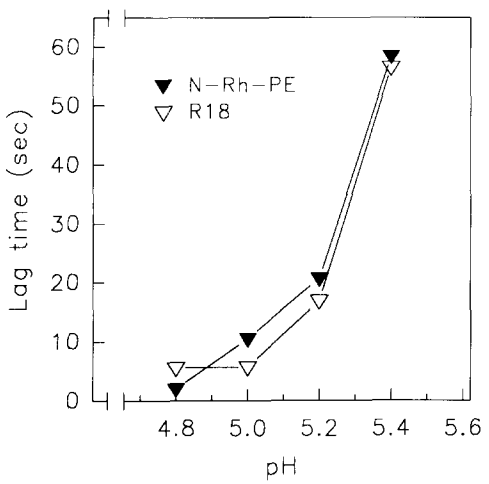

Fig. 5. pH dependence of the delay time of the onset of virus fusion with erythrocyte membranes after lowering the pH at 20°C. Influenza virus was labelled either with R18 or with N-Rh-PE and bound to erythrocyte membranes. Delay times were obtained from kinetics of fluorescence dequenching measured as described in legend to Fig. 4 (time resolution 0.5 s).

observed a pH and temperature dependent lag of the onset of fusion after lowering the pH (Fig. 5). No difference of the lag between R18 and N-Rh-PE labelled viruses was observed.

In conclusion, these results suggest that neither the type of fluorophore nor the labelling protocol affect the extent and kinetics of low pH-induced fusion of influenza virus measured by fluorescence dequenching (see also below) when used in a sensible concentration range.

Virus-induced haemolysis

To sustain further our conclusion that the labels used did not affect virus-target membrane interaction

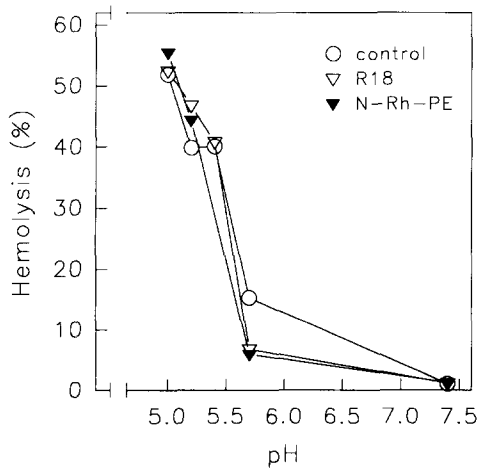

Fig. 6. pH dependence of influenza virus A/PR 8/34 induced haemolysis of RBC. ○, control (non-labelled virus); ▽, R18 labelled virus; ▼, N-Rh-PE labelled virus. Haemolysis was measured 5 min after lowering to the indicated pH, 37°C, as described in Materials and methods.

we have measured the virus-induced haemolysis of intact red blood cells at low pH and 37°C. Haemolytic activity correlates with the HA-associated fusogenic properties of influenza virus. Eg, inactivation of influenza virus by low pH pretreatment inhibits both fusion and haemolysis. As can be seen (Fig. 6) no differences in haemolysis were established between unlabelled (control) and labelled viruses (R18, N-Rh-PE).

4. Discussion

Membrane mixing assays utilizing fluorescent membrane markers have become the most employed method to monitor fusion of intact enveloped viruses with target membranes. Mainly those techniques are applied which are based on fluorescence dequenching of lipid-like probes incorporated into the virus membrane. One of the critical assumptions underlying this assay that it is insensitive to a simple aggregation of membranes. In the present study we addressed the possibility of artifacts arising from probe exchange without lipid mixing by fusion. To this end we have compared the fluorescence dequenching upon low pH-triggered fusion of influenza virus membrane using two different markers incorporated into the lipid phase of influenza virus membrane, the 'lipid-like' fluorophore R18 and the fluorescent phospholipid analogue N-Rh-PE. For both markers we observed the same kinetics as well as the same final extent of fluorescence dequenching upon fusion. Since N-Rh-PE with its two long hydrophobic fatty acid residues is known to be strongly intercalated into biological membranes [7–9], rates of non-specific dequenching would be very unlikely to be the same for both fluorophores. Thus, we can conclude that the dequenching of either fluorophore is not caused by non-specific lipid exchange. This is strengthened by the observation, that incubation of virus-cell complexes at neutral pH did not result in any fluorescence enhancement. Moreover, for both labels no dequenching was found at low pH when the virus was inactivated prior to binding to erythrocyte membranes. Likewise, haemolysis of R18 or N-Rh-PE labelled viruses at low pH were similar to the control (unlabelled viruses). This suggests that both membrane markers can be used to monitor influenza virus fusion since (i) they do not affect fusogenic properties of influenza virus in the concentration range investigated and (ii) no non-specific marker transfer occurs.

Although the data clearly show that dequenching of R18 reflects membrane fusion it is worth noting that the assay based on N-Rh-PE requires a lower membrane concentration of the fluorophore. Self-quenching of N-Rh-PE in virus membrane is more efficient com-

pared to R18. The mechanism of self-quenching of R18 and N-Rh-PE in virus membranes is beyond the scope of this study. However, the temperature dependence of fluorescence intensity implies that for both fluorophores some static mechanism might be involved in self-quenching. Thus, one may surmise that fluorophores form small aggregates which become instable by increasing the temperature. Macdonald [21] has investigated in more detail self-quenching of R18 in liposomal bilayers suggesting that no collisional mechanism is responsible for quenching. Although further investigations are required she proposed that energy transfer without emission to nonfluorescent dimers is involved in self-quenching of R18.

The more efficient self-quenching of N-Rh-PE with respect to R18 in influenza virus membranes can be partly attributed to the pronounced transversal asymmetric distribution of N-Rh-PE in the virus membrane. From a modified Stern-Volmer-plot of fluorescence quenching by the non-permeable aqueous quencher KJ we concluded that the fluorescent lipid analogue is exclusively located on the outer leaflet. This is not surprising since it has been shown that the passive flip-flop of phospholipids in virus membranes is very slow [22;23]. Moreover, it has been revealed that the transversal motion of N-Rh-PE in biological membranes is very slow, e.g., in red blood cell membranes [24]. Therefore, it is reasonable to assume that in the time frame of our experiments the probe remains in the outer leaflet upon its incorporation. Surprisingly, as deduced by KJ quenching R18 seems to be almost equally distributed between both leaflets of influenza virus. This implies that R18 can rather easily traverse the virus membrane. It has been shown that substances with a hydrophobic acyl chain and a polar head like fatty acids can undergo very fast flip-flop in membranes [25]. Our observation of a randomized distribution of R18 in influenza virus membrane is different from that of Hoekstra et al. [1] who suggested for Sendai virus membranes that R18 resides mainly in the outer layer. They have found that self-quenching of R18 in Sendai virus is much stronger than in liposomes with a randomized distribution of the fluorophore. However, Aroeti and Henis [2] gave evidence that efficient self-quenching of R18 in Sendai virus membranes is attributed mainly to its distribution in distinct lipid domains rather than to transversal inhomogeneous distribution of R18 or specific interaction with viral membrane proteins. According to recent investigations of MacDonald [21] cholesterol or any membrane-condensing lipid might be involved in the enhancement of R18 selfquenching. Perhaps, a similar mechanism has to be taken into account in efficient selfquenching of N-Rh-PE in influenza virus. From Fig. 1 it can be concluded that the asymmetric distribution of N-Rh-PE cannot explain solely the dependence of selfquenching on the membrane concentration of the fluorophore. Further investigations are warranted to clarify this point.

To sum up, our data confirm previous observations [1] that the fluorophore R18 is a useful marker to assess the kinetics and the extent of influenza virus fusion. It does not affect the fusogenic properties at low label concentration which are still sufficient to monitor fusion in a quantitative manner. Moreover, we have no evidence for non-specific exchange of the fluorophore interfering with the fusion process between virus and appropriate target membranes. Our results are in disagreement with those of Wunderli-Allensbach et al. [4] who observed marker exchange between non-fused membranes and an impairment of fusogenic properties. Surely, at rather high membrane concentrations of fluorophore one should expect an inactivation of virus infectivity. Membrane concentrations up to 1.4 R18 molecules per nm^2 membrane surface have been used in that report [4]. This is rather high assuming that the cross sectional area of R18 is in the order of phospholipid head groups (~ 0.6 nm^2 per head group).

5. Acknowledgments

We are indebted to Dr. M. Clague (EMBL, Heidelberg) for helpful comments. A.H. was supported by grants from the Deutsche Forschungsgemeinschaft (He1928/1-1; SFB 312 D9/YE3) and the Fritz-Thyssen-Stiftung. T.K. is a recipient of a fellowship from the Boehringer-Ingelheim-Fonds.

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