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The influenza virus-induced fusion of erythrocyte ghosts does not depend on osmotic forces

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The role of osmotic forces and cell swelling in the influenza virus-induced fusion of unsealed or resealed ghosts of human erythrocytes was investigated under isotonic and hypotonic conditions using a recently developed fluorescence assay (Hoekstra, D., De Boer, T., Klappe, K., Wilschut, J. (1984) Biochemistry 23, 5675–5681). The method is based on the relief of fluorescence selfquenching of the fluorescent amphiphile octadecyl rhodamine B chloride (R_{18}) incorporated into the ghost membrane as occurs when labeled membranes fuse with unlabeled membranes. No effect neither of the external osmotic pressure nor of cell swelling on virally mediated ghost fusion was established. Influenza virus fused unsealed ghosts as effectively as resealed ghosts. It is concluded that neither osmotic forces nor osmotic swelling of cells is necessary for virus-induced cell fusion. This is supported by microscopic observations of virus-induced fusion of intact erythrocytes in hypotonic and hypertonic media. A disruption of the spectrin-actin network did not cause an enhanced cell fusion at acidic pH of about 5 or any fusion at pH 7.4.

Introduction

The molecular mechanisms involved in biological membrane fusion processes are currently a central subject of membrane research because fusion is a crucial, intermediate step in many biological phenomena e.g. fertilization, endo- and exocytosis, and myoblast fusion. In earlier membrane fusion models much attention was given to the structure and physical properties of membranes and molecules involved in fusion (for a review, see Wilschut and Hoekstra [1]). However, the forces responsible for the required destabilization of two closely apposed membranes to be fused are unknown.

Recently, Lucy and Ahkong [2] proposed that the involvement of osmotic forces may be a general feature of fusion reactions of biological membranes. They suggested that fusion of biological membranes requires perturbed phospholipid bilayers being forced into a single bilayer, which is then ruptured. Both events being driven by osmotic forces. Lucy and Ahkong [2] take special emphasis on re-interpretation of observations on enveloped virus induced membrane fusion in terms of their model. They suggested that fusion of erythrocytes by haemolytic Sendai virus is started by virus-cell fusion which permeabilises the erythrocyte plasma membrane. Osmotic swelling of cells then drives the fusion of the erythrocytes with one another. Recently further indications for an essential role

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of osmotic forces in virally induced membrane fusion came from the group of Loyter [3,4]. They suggested that osmotic swelling of recipient cells subsequent to a virus induced increase of cell membrane permeability leads to an exposure of membrane phospholipids to the action of the viral glycoproteins, and afterwards, to membrane fusion.

However, there is also contradictory evidence. Sekiguchi et al. [5] reported that osmotic cell swelling is not required for Sendai virus-induced erythrocyte ghost fusion. Knutton and Bächi [6] observed fusion events using unsealed ghosts as a target for Sendai viruses.

In an previous paper we could demonstrate that influenza virus-ghost fusion is independent of osmotic forces as well as osmotic swelling [7]. To clarify further the contradictory results on the role of osmotic phenomena in virally induced cell membrane fusion we investigated the influence of osmotic pressure of the suspension medium on the influenza virus-induced cell-cell fusion using human erythrocytes as target membranes. Since influenza virus induces cell fusion only at acidic pH whereas virus adsorption and virally induced cell aggregation also take place at neutral pH [8,9] this fusion system offers the opportunity to test if osmotic forces can nucleate fusion of aggregated cells (at neutral pH) and/or can enhance and accelerate fusion process (at pH of about 5).

Besides light microscopic observations we used a recently developed fusion assay [10,11], which is based on the insertion of the fluorescent amphiphile octadecyl Rhodamine B chloride (R_{18}) at a selfquenching concentration into erythrocyte ghost membranes. Influenza virus-induced fusion between labeled and unlabeled ghost membranes is accompanied by a dilution of the probe, and hence by an increase of the fluorescence which is a direct measure of fusion. The main result of this study is that virally induced fusion of erythrocyte ghosts requires neither osmotic forces nor cell swelling.

Materials and Methods

Erythrocyte and ghost preparation

Human erythrocytes (O Rh^+) from different donors were used within 7 days after sampling (blood bank, ACD storage medium, 4°C). After centrifugation at $500 \times g$ the erythrocytes were washed twice at $2000 \times g$ with 5 vol. of isotonic NaCl: 150 mM NaCl, 5.8 mM phosphate buffer (pH 7.4) or 150 mM NaCl, 20 mM sodium acetate buffer (pH 5.2).

Resealed human erythrocytes ghosts were prepared according to Steck and Kant [12] with the modifications described by Hoekstra et al. [13], except that the lysed cells were resuspended in isotonic NaCl (pH 7.4), which was also used for washing procedure of the ghosts.

Unsealed erythrocyte ghosts were prepared according to Dodge et al. [14].

Protein concentration of ghosts as well as of viruses was determined according to the method of Lowry et al.

Ghosts were labeled with R_{18} (Molecular Probes) as described by Hoekstra et al. [10]. Ghost were incubated in the dark (room temperature) for 1 h at a label concentration of 15 μg R_{18}/mg ghost protein.

Virus preparation

Influenze virus A/PR8/34 were kindly supplied by Zentralinstitut für Hygiene, Mikrobiologie und Epidemiologie, Berlin.

They were grown for 3 days in the allantoic cavity of 11-day-old embryonated eggs, precipitated with saturated $(NH_4)_2SO_4$ solution, purified by centrifugation in phosphate buffer and stored at 4°C.

The extent of virus-mediated cell-cell fusion at a given virus protein concentration was different for various virus preparations.

Light microscopic observation of erythrocyte fusion

Intact erythrocytes (hematocrit of 2.5% or 0.5%) were incubated with various amounts of influenza virus at 4°C for 10 min. After incubating the suspension at 37° (30 min) the particles were counted using phase contrast microscopy ensuring also counting of ghosts. The fusion index [15,16]

$$\text{fusion index} = \frac{\text{particle count in control}}{\text{particle count after fusion}} - 1$$

was calculated.

To elucidate the effect of osmotic swelling erythrocytes were also incubated in hypotonic (100

mM NaCl, 20 mM sodium acetate (pH 5.2)) or hypertonic (150 mM NaCl, 20 mM sodium acetate, 150 mM raffinose (pH 5.2)) media. Alternatively, also mixtures of buffers A/B and C/D, (see below), respectively, were used to establish hypotonic conditions.

Virally induced fusion of R_{18}-labeled ghosts with unlabeled ghosts

The following buffers were used: (A) 132 mM NaCl, 35 mM sucrose, 20 mM sodium acetate (pH 5.2), π (osmotic pressure) = 326 mosmol; (B) 20 mM sodium acetate. If not stated otherwise the pH of buffers A and B was adjusted to 5.2. (C) 150 mM NaCl, 5.8 mM phosphate buffer (pH 7.4), π = 304 mosmol; (D) 5.8 mM phosphate buffer (pH 7.4). Osmotic pressure was determined with a Knauer osmometer at room temperature.

2 ml of the corresponding buffer were mixed with 10 μl unlabeled ghosts (7–10 mg protein/ml), 10 μl labeled ghosts (labeled ghosts/unlabeled ghosts = 1:6) and 10 μl virus suspension (final concentration 30 μg protein/ml) and kept at 0°C for 10 min for virally induced ghost aggregation. The mixture was warmed to 37°C within 15 s and immediately transferred to a prewarmed quarz tube used for fluorescence measurement. Fusion was monitored as described [7,10,11] by measuring fluorescence dequenching at excitation and emission wavelengths of 560 nm and 590 nm, respectively, using a Perkin-Elmer MPF-44B spectrophotofluorometer equipped with a chart recorder and a thermostated sample holder. The scale was calibrated such that the residual fluorescence of R_{18}-labeled ghosts was taken as zero level and the fluorescence obtained after addition of Triton X-100 (50 μl 20% v/v), corrected for sample dilution, as 100% (infinite dilution).

For fusion experiments under hypotonic conditions the external osmotic pressure was decreased by mixing isotonic buffer (A or C) and hypotonic buffer (B or D) in different ratios. Labeled, unlabeled ghosts and virus were treated as described except that all steps were done in the desired hypotonic media, or they were incubated in a definite volume of isotonic buffer at 0°C for 10 min and 30 s after shifting the temperature to 37°C prewarmed hypotonic buffer was added to give a final volume of 2 ml.

The rate constant k_F of the fusion kinetic was estimated by fitting the fluorescence tracings to a first-order kinetic. The extent of fusion $F(t)$ at the time t is given by:

$$F(t) = F(t=\infty) * (1 - \exp[-k_F * t]) \quad (1)$$

$F(t = \infty)$ corresponds to the final level of fusion. Fitting was performed by nonlinear regression using the nonlinear least-squares program (MULTI) [17]. The curves could be fitted quite reasonable to the model given (Eqn. 1). In all cases the nonlinear coefficient of determination [18] was not smaller than 0.96.

Statistical comparison was done by using the t-test (paired observations, $\alpha = 0.05$).

Results

A. Fluorescence assay

Characteristic of influenza virus-induced cell-cell fusion

In Fig. 1 a typical kinetic of influenza virus-induced fusion of unlabeled and labeled resealed ghosts is compared with that of the fusion of labeled influenza virus with resealed ghosts. Obviously, the fluorescence intensity has already reached its plateau phase almost after 10 min at 37°C. The increase of fluorescence intensity due to dilution of R_{18} into unlabeled membranes is directly proportional to fusion extent under these conditions [10,11]. Therefore, the fusion values given in the following refer to the increase of fluorescence after 10 min at 37°C.

The extent of virus-mediated ghost fusion was between 30 and 50% after 10 min at 37°C (pH 5.2). The fluorescence increase in the absence of virus or unlabeled ghosts did not exceed 5% after 10 min at 37°C (pH 5.2).

The fusion extent was not affected by pH (t-test, $\alpha = 0.05$) in the range investigated (pH 4.8–5.2). However, the rate constant k_F at pH 5.2 (2.18 ± 0.32/min ($n = 3$), average ± S.D.) and at pH 5.0 (2.10 ± 0.29/min ($n = 3$)) differs significantly from that at pH 4.8 (1.36 ± 0.42/min ($n = 4$)). The rate constant of the fusion between labeled A/PR8/34 virus with ghosts is about three times higher (k_F

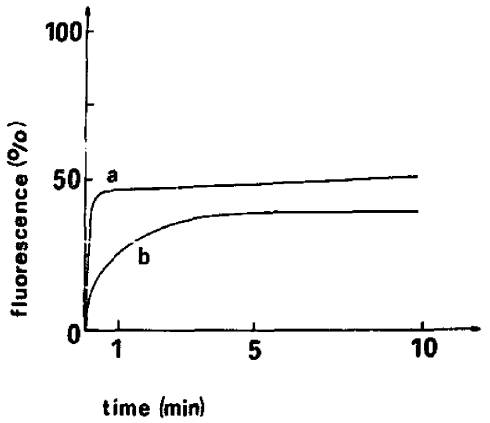

Fig. 1. Kinetic of fusion of R_{18}-labeled influenza virus (A/PR8/34) with resealed erythrocyte ghosts (a) and influenza virus mediated fusion of R_{18}-labeled resealed ghosts with unlabeled ghosts (b) at pH 4.8 (37°C). Virally mediated ghost fusion was performed as described in Materials and Methods. Fusion of R_{18}-labeled virus with ghosts was done by mixing 20 μl of a ghost suspension (approx. 6 mg ghost protein/ml) and 10 μl of a stock solution of labeled virus (approx. 1 mg virus protein/ml) in 2 ml of buffer A. After keeping the suspension at 0°C for 5 min the mixture was warmed to 37°C within 15 s and immediately transferred to a prewarmed tube used for fluorescence measurements. Influenza virus was labeled as described recently [7,10,11].

$= 6/\text{min}$} (cf. also Ref. 7) (Fig. 1) than k_F of virally induced ghost-ghost fusion.

No significant differences between the fusion characteristic of unsealed and resealed ghosts were observed under these conditions (not shown), indicating that osmotic swelling of the cells is not a prerequisite to initiate virally induced cell fusion or virus cell fusion [7].

Influence of external osmotic pressure on virally induced ghost-ghost fusion

Often albumin is incorporated into resealed ghosts to allow for swelling in isotonic media under certain conditions. We avoid this technique since a binding of the virus bound label to albumin during virus-cell fusion would result in an additional fluorescence dequenching which is not due to dilution of the label within the cell membrane.

To investigate whether osmotic forces and cell swelling may increase the percentage of fusion, the external osmotic pressure was decreased before or 30 s after transferring the sample to 37°C (cf. Materials and Methods).

Lowering the osmotic pressure from 326 mosmol to 233 mosmol by dilution of buffer A with buffer B in a ratio of 2:1 did not affect the fusion extent independent of the ghost type used (Table I). It also turned out that the rate constant k_F of ghost-ghost fusion in hypotonic suspension media was not significantly different in comparison to the control (isotonic conditions); e.g. $k_F = 2.18 \pm 0.32/\text{min}$ ($n = 3$) in isotonic media and $k_F = 2.48 \pm 0.42/\text{min}$ ($n = 3$) in hypotonic media at pH 5.2 (average ± S.D.).

After decreasing the osmotic pressure to 188 mosmol by a 1:1 dilution of buffer A with buffer B we observed a significantly raised fluorescence

TABLE I

EFFECT OF EXTERNAL OSMOTIC PRESSURE ON THE EXTENT OF INFLUENZA VIRUS MEDIATED FUSION OF R_{18}-LABELED RESEALED GHOSTS WITH UNLABELED GHOSTS AT DIFFERENT pH VALUES

The percentage of fusion was measured by the fluorescence intensity reached after 10 min at 37°C. The external osmotic pressure was lowered before (II) or 30 s after (III) raising the temperature of the suspension to 37°C by mixing buffer A with buffer B in a ratio of 2:1 ($\pi = 233$ mosmol). (I) refers to the control sample in buffer A ($\pi = 326$ mosmol, cf. Materials and Methods). The average and the standard deviation of three independent measurements ($n = 3$) are given.

Sample	Osmotic pressure π (mosmol)	Fusion extent (%)		
		pH of the suspension medium		
		4.8	5.0	5.2
I, control	326	38.1 ± 3.5	44.2 ± 3.4	39.6 ± 2.2
II, π was lowered before incubating at 37°C	233	40.6 ± 3.1	43.3 ± 4.3	39.9 ± 3.5
III, π was lowered 30 s after raising the temperature to 37°C	233	38.5 ± 3.3	41.4 ± 2.9	38.0 ± 2.8

TABLE II

EFFECT OF EXTERNAL OSMOTIC PRESSURE ON THE EXTENT OF INFLUENZA VIRUS MEDIATED FUSION OF R_{18}-LABELED RESEALED GHOSTS WITH UNLABELED GHOSTS AT DIFFERENT pH VALUES

The percentage of fusion was measured by the fluorescence intensity reached after 10 min at 37°C. The external osmotic pressure was lowered 30 s after (II) raising the temperature of the suspension to 37°C by mixing buffer A with buffer B in a ratio of 1:1 (π = 188 mosmol). (I) refers to the control sample in buffer A (π = 326 mosmol, cf. Materials and Methods). The average and the standard deviation of three independent measurements (n = 3) are given.

Sample	Osmotic pressure π (mosmol)	Fusion extent (%)		
		pH of the suspension medium		
		4.8	5.0	5.2
I, control	326	26.1 ± 3.3	24.2 ± 3.3	30.0 ± 3.1
II, π was lowered 30 s after raising the temperature to 37°C	188	35.6 ± 2.6	39.6 ± 2.1	43.2 ± 1.6

intensity (t-test; $\alpha = 0.05$) in comparison to the control (only buffer A) for resealed ghosts (Table II). Similar results were obtained by using unsealed ghosts (e.g. 25.5 ± 3.2% (n = 3) at 326 mosmol and 40.9 ± 3.6% (n = 3) at 188 mosmol, pH 5.2). This increase is not due to an enhanced virally induced ghost fusion because performing these experiments in the absence of virus (no ghost-ghost fusion) a fluorescence dequenching of about 10% was observed after 10 min at 37°C (data not shown). This value is almost equal to the increase of fluorescence dequenching (about 10–15%, Table II) in hypotonic media (1:1 dilution).

The reason for this fluorescence increase in comparison to the control remains unclear. The higher fluorescence intensity in hypotonic media (1:1 dilution) is not due to a lower NaCl-concentration, since fusion was not affected by such a low ionic concentration in comparison to control sample if isotonicity was restored by sucrose (data not shown). It may be that under certain conditions the asymmetric distribution of R_{18} in ghost membranes [10,11] is disturbed leading to a decrease of fluorescence dequenching.

Similar investigations with both types of ghosts were done at pH 7.4 where virus-mediated aggregation but no fusion occurs. The fluorescence signal remained nearly constant in buffer C (304 mosmol) at 37°C even in the presence of adsorbed virus. Only a slight increase (≈ 5%) of the fluorescence signal was found after 10 min at pH 7.4 which was also observed in the absence of unlabeled ghosts. However, independent of the ghost type used, under no circumstances any evidence for a fusion induced by lowering the external osmotic pressure was obtained (mixing buffer C with buffer D in the ratio of 2:1 (225 mosmol) or 1:1 (189 mosmol); not shown). This was also confirmed by preincubating ghosts at 50°C for 5 min in order to denature spectrin [19] which is the main component of the erythrocyte cytoskeleton. Comparable to the results at pH 5.2 an enhanced dequenching was observed in hypotonic media (1:1 dilution of buffer C with D) also in the absence of virus.

B. Light microscopy investigations of virally induced erythrocyte fusion

The fusion index of virally induced fusion of intact erythrocytes (pH 5.2) was determined at different virus concentrations. A preincubation of erythrocytes for 5 min at 50°C resulted in a significantly lowered fusion index at higher virus concentrations (Fig. 2). No significant increase of the numbers of fused cells was observed by lowering the external pressure (up to 188 mosmol) before or 1 min after transferring the cells to 37°C independent of the medium used (cf. Materials and Methods, example given in Fig. 2). In contrast, a significantly lowered fusion index (t-test; $\alpha = 0.05$) was observed under hypertonic conditions raising the osmotic pressure by addition of 150 mM raffinose (final concentration) to the suspension medium of 150 mM NaCl, 20

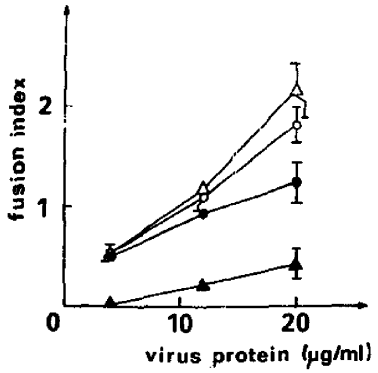

Fig. 2. Influenza (A/PR8/34) virus-induced fusion of human erythrocytes at pH 5.2 under isotonic (○, ●), hypotonic (△) (100 mM NaCl, 20 mM sodium acetate), hypertonic (▲) (150 mM NaCl, 150 mM raffinose, 20 mM sodium acetate) conditions and after preincubation of erythrocytes at 50 °C (5 min) (●). Fusion index was determined after 30 min at 37 °C. The average and the standard estimate of error of at least three independent measurements are presented.

mM sodium acetate (pH 5.2). However, removal of raffinose by washing (buffer C) the erythrocyte suspension after 30 min at 37 °C and a further incubation at 37 °C led to an increase of fusion index (not shown) comparable to that of the control sample.

Performing the same experiments at pH 7.4 under no circumstances a fusion of erythrocytes was observed. Also preincubation of cells at 50 °C (5–10 min) or allowing virus adsorption and virus-induced cell aggregation at pH 5.2 (4 °C) did not cause any fusion at pH 7.4 (37 °C) in isotonic and hypotonic media.

Discussion

The main purpose of this paper was to elucidate the role of osmotic forces and cell swelling in virus-mediated cell fusion using different types of erythrocyte ghosts. Resealed ghosts have similar permeability and transport properties [20,21] as well as osmotic response [20,22] as erythrocytes. Therefore, they may serve as osmotically active cells whereas unsealed ghosts do not show any osmotic response.

As has been shown by the fluorescence assay the influenza virus-induced ghost fusion at a pH of about 5 is as effective with unsealed ghosts as with resealed ghosts independent of the experimental conditions. Furthermore, no enhanced fusion extent of resealed ghosts was observed in hypotonic media lowering the osmotic pressure up to about 190 mosmol at various steps of the experimental procedure. Therefore, it can be concluded that influenza virus-mediated fusion of ghost membranes is independent of osmotic forces.

Loyter and co-workers [3,4] suggested that osmotic swelling of recipient cells leads to exposure of naked membrane phospholipids to the action of viral glycoproteins. Our results did not support this hypothesis since allowing resealed ghosts to swell before starting fusion at 37 °C did not result in a higher percentage of fusion. In this case it can be assumed that the resealed ghosts are in osmotic equilibrium when starting fusion due to the very high water permeability of erythrocyte membranes [23].

Also creating an osmotic nonequilibrium during virally induced fusion of resealed ghosts did not effect the fusion extent. This was shown by lowering the external osmotic pressure 30 s after transferring the suspension to 37 °C.

Light microscopic investigations of influenza virus-mediated fusion of intact erythrocytes at a pH of about 5 sustain the conclusion that osmotic forces are not involved in virally induced ghost membrane fusion. Under no circumstances hypotonic osmotic pressure raised the fusion extent. In contrast, the number of fused cells was significantly lower in hypertonic media after incubation at 37 °C for 30 min. However, the fusion index was comparable to the control sample after removal of the trisaccharide raffinose. Since the fusion capacity of influenza virus is diminished after such a long incubation in acidic media at 37 °C [24,25] it can be concluded that the virally induced erythrocyte fusion took place also under conditions where cell swelling is hampered due to the presence of an osmotically active substance of higher molecular weight in the suspension medium. Swelling is a prerequisite for rounding of fused cells and the detection of polyghosts in the light microscopy which is only an indirect measure of cell-cell fusion [5].

These results did not support the membrane fusion model of Lucy and Ahkong [2] emphasizing the essential role of osmotic forces in membrane fusion. It turned out from our investigations that

the unique properties of the HA glycoprotein are responsible for inducing cell-cell fusion. This is underlined by the failure to show any virally induced ghost fusion at pH 7.4 under isotonic as well as hypotonic conditions although ghosts are aggregated by the virus. There are several lines of evidence that the irreversible conformational change and protonation of the HA protein at acidic pH is the key step in the activation of the fusion capacity of the virus [24,26]. A apolar segment (HA_2 subunit) which is exposed after the conformational change would penetrate into the target membrane, thus creating the nucleation point for fusion by a local perturbation of the membrane structure [26,27].

Ahkong and Lucy [28] gave evidence that a possible reason for the failure of erythrocytes to fuse when they are subjected to osmotic swelling alone is the restraining influence of their membrane skeleton. They observed on Alcian blue-coated plastic dishes a fusion of densely packed hen erythrocytes in hypotonic media after preincubation of the cells at 50 °C. This treatment is known to disrupt the spectrin-actin network [16]. However, recently we reported that the fusion between R_{18}-labeled influenza virus and ghosts [7] is lowered after preincubation of ghosts at 50 °C. Also our light microscopy observation presented suggests that virally induced fusion of intact erythrocytes is not enhanced at a pH of about 5 by preincubation of red blood cells at 50 °C. It may be that the lowered deformability of erythrocyte membranes after heat denaturation of spectrin [29] is responsible for the less effective virally mediated fusion of erythrocytes at higher virus concentrations (Fig. 2) as well as for the lowered virus-cell fusion [7]. Heat treatment of erythrocytes did not cause fusion in isotonic and hypotonic media at pH 7.4. A similar result was reported by Baker and Clark [30]. They found that calcium phosphate-induced fusion of intact erythrocytes was inhibited when cells were heated to 48–50 °C.

Our conclusions are in accordance with that of Knutton and Bächi [6] and Sekiguchi et al. [5] that the primary virally induced fusion between ghost membranes is independent of osmotic forces but rounding and polyghost formation following cell-cell fusion are osmotically driven processes.

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References

1 Wilschut, J. and Hoekstra, D. (1984). Trends Biochem. Sci. 9, 479–483.
2 Lucy, J.A. and Ahkong, Q.F. (1986) FEBS Lett. 199, 1–11.
3 Loyter, A., Citovsky, V. and Nussbaum, O. (1987) Summary W7 Int. Symp. on Molecular Mechanisms of Membrane Fusion, Buffalo 1986, p. 26.
4 Citovsky, V. and Loyter, A. (1985) J. Biol. Chem. 260, 12072–12077.
5 Sekiguchi, K., Kuroda, K., Ohnishi, S.-I. and Asano, A. (1981) Biochim. Biophys. Acta 645, 211–225.
6 Knutton, S. and Bächi, T. (1980) J. Cell. Sci. 42, 153–167.
7 Pritzen, C. and Herrmann, A. (1988) Biosci. Rep. 8, 55–64.
8 Maeda, T. and Ohnishi, S.-I. (1980) FEBS Lett. 122, 283–287.
9 Maeda, T., Kawasaki, K. and Ohnishi, S.-I. (1981) Proc. Natl. Acad. Sci. USA 78, 4133–4137.
10 Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) Biochemistry 23, 5675–5681.
11 Hoekstra, D., Klappe, K., De Boer, T. and Wilschut, J. (1985) Biochemistry 24, 4739–4745.
12 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31, 172–180.
13 Hoekstra, D., Wilschut, J. and Scherphof, G. (1985) Eur. J. Biochem. 146, 131–140.
14 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130.
15 Peretz, H., Toister, Z., Laster, Y. and Loyter, A. (1974) J. Cell. Biol. 63, 1–11.
16 Donath, E., Herrmann, A., Coakley, W.T., Groth, Th., Egger, M. and Taeger, M. (1987) Biochem. Pharmacol. 36, 481–487.
17 Yamaoka, K., Tanigawara, Y., Nakagawa, T. and Uno, T. (1981) J. Pharm. Dyn. 4, 879–885.
18 Sachs, A. (1978) Angewandte Statistik, p. 346, Springer-Verlag, Berlin, Heidelberg and New York.
19 Brandts, J.F., Erickson, L., Lysko, K., Schwartz, A.T. and Taverna, R.D. (1977) Biochemistry 16, 3450–3454.
20 Jausel-Hüsken, S. and Deuticke, B. (1981) J. Membr. Biol. 63, 61–70.
21 Müller, P., Herrmann, A., Bernhardt, I. and Glaser, R. (1985) Biosci. Rep. 5, 425–432.
22 Chang, T., Sharpless, T.B., Davenport, D.A., Radunsky, M.B. and Yu, H. (1983) Biochim. Biophys. Acta 731, 346–353.
23 Conlon, T. and Outhred, R. (1978) Biochim. Biophys. Acta 411, 408–413.
24 Sato, S.B., Kawasaki, K. and Ohnishi, S.-I. (1983) Proc. Natl. Acad. Sci. USA 80, 3153–3157.

25 Stegmann, T., Hoekstra, D., Scherphof, G. and Wilschut, J. (1986) J. Biol. Chem. 261, 10966–10969.
26 White, J., Kielian, M. and Helenius, A. (1983) Q. Rev. Biophys. 16, 151–195.
27 Doms, R.W., Helenius, A. and White, J. (1985) J. Biol. Chem. 260, 2973–2981.
28 Ahkong, Q.F. and Lucy, J.A. (1986) Biochim. Biophys. Acta 858, 206–216.
29 Heath, B.P., Mohandas, N., Wyatt, J.L. and Shohet, S.B. (1982) Biochim. Biophys. Acta 691, 211–219.
30 Baker, R.F. and Clark, L.J. (1979) Biorheology 16, 285–292.