Fusion between Newcastle disease virus and erythrocyte ghosts using octadecyl Rhodamine B fluorescence assay produces dequenching curves that fit the sum of two exponentials

César COBALEDA, Adolfo GARCÍA-SASTRE and Enrique VILLAR*

Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Salamanca, Avda. Campo Charro, s/n, 37007 Salamanca, Spain

The kinetics of fusion between Newcastle disease virus and erythrocyte ghosts has been investigated with the octadecyl Rhodamine B chloride assay [Hoekstra, De Boer, Klappe, and Wilschut (1984) Biochemistry **23**, 5675–5681], and the data from the dequenching curves were fitted by non-linear regression to currently used kinetic models. We used direct computer-assisted fitting of the dequenching curves to the mathematical equations. Discrimination between models was performed by statistical analysis of different fits. The experimental data fit the exponential model previously published [Nir, Klappe, and Hoekstra (1986) Biochemistry **25**, 2155–2161] but we describe for the first time that the best fit was achieved for the sum of two exponential terms: $A_1[1-\exp(-k_1t)]+A_2[1-\exp(-k_2t)]$. The first exponential term represents a fast reaction and the second a slow dequenching reaction. These findings reveal the existence of two independent, but simultaneous, processes during the fusion assay. In order to challenge the model and to understand the meaning of both equations, fusion experiments were carried out under different conditions well known to affect viral fusion (changes in pH, temperature and ghost concentration, and the presence of disulphide-reducing agents or inhibitors of viral neuraminidase activity), and the same computer fitting scheme was followed. The first exponential equation represents the viral protein-dependent fusion process itself, because it is affected by the assay conditions. The second exponential equation accounts for a non-specific reaction, because it is completely independent of the assay conditions and hence of the viral proteins. An interpretation of this second process is discussed in terms of probe transfer between vesicles.

INTRODUCTION

Newcastle disease virus (NDV) is an enveloped virus belonging to the family of Paramyxoviridae. In the viral infection cycle, the fusion between the viral membrane and that of the susceptible cell is an essential step in the uncoating and penetration of the virus (Choppin and Compans, 1975; Rott, 1979; Choppin and Scheid, 1980; White, 1990; Galinski and Wechsler, 1991; Morrison and Portner, 1991). NDV, and other paramyxoviruses, have two glycoproteins: the haemagglutinin neuraminidase (sialidase) (HN) and the fusion (F) protein, associated with the viral membrane projecting from the external surface of the lipid envelope (Choppin and Compans, 1975; Choppin and Scheid, 1980). These are transmembrane proteins, extending through the lipid bilayer and exposing a segment of the polypeptide to the internal surface of the envelope (Lyles, 1979; Morrison, 1988). The fusion of paramyxoviruses, such as NDV, with host cell starts with the adsorption of the virus to the target plasma cell membrane. This process is mediated by the virus-attachment protein, the HN glycoprotein, through its haemagglutinin activity. After attachment to cell receptors, the F protein, consisting of two disulphide-linked subunits, F_1 and F_2, mediates virus–cell fusion by directly interacting with the cell membrane (Choppin and Schied, 1980; Galinski and Wechsler, 1991), at neutral pH (Lorge et al., 1986; White, 1992). The F protein also mediates syncytium formation by promoting fusion between an infected cell and an adjacent cell (Morrison and Portner, 1991). The role of HN in membrane fusion is still controversial. Some data support the idea that it might somehow participate in the fusion process. It has been shown (Citovski and Loyter, 1985; Citovski et al., 1986) that both HN and F protein have to be active and within the same membrane particle in order to achieve fusion and that the NDV HN glycoprotein cannot be replaced by influenza virus HA glycoprotein in the fusion process (Morrison et al., 1991). In the same way, the presence of sialic acids in plasma-membrane receptors seems to be necessary for other paramyxoviruses to fuse with cells (Moscona and Peluso, 1991). Besides, fusion can be blocked by anti-HN monoclonal antibodies (Miura et al., 1982; Iorio et al., 1992), although it is also possible that the receptor-binding domain of HN is distinct from the haemagglutinating domain (Galinski and Wechsler, 1991) and even the presence of a specific site on the HN glycoprotein to interact with the F glycoprotein for the fusion process is possible (Iorio et al., 1992).

The fusion process can be studied by a fluorescence assay, which allows monitoring of virus–cell fusion in a direct and continuous manner (Hoekstra et al., 1984), by inserting the fluorescent dye octadecyl Rhodamine B chloride (R_{18}) into the viral membrane. This procedure has now been widely used to monitor virus–cell fusion using Sendai virus (Hoekstra et al., 1985, 1989; Hoekstra and Klappe, 1986; Nir et al., 1986a,b, 1990; Klappe et al., 1986; Pedroso de Lima et al., 1991, 1992), NDV (Lorge et al., 1986), influenza virus (Morris et al., 1989; Wunderli-Allenspach et al., 1990; Wunderli-Allenspach and Ott,

Abbreviations used: DTT, dithiothreitol; HA, influenza virus haemagglutinin; HN, Newcastle disease virus haemagglutinin neuraminidase protein; KNP, 120 mM KCl/30 mM NaCl/10 mM sodium phosphate buffer, pH 7.4; Neu5Ac2en, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid; NDV, Newcastle disease virus; R_{18}, octadecyl Rhodamine B chloride; FDQ, fluorescence dequenching; %FDQ*t*, percentage of fluorescence dequenching at a given time.

* To whom correspondence should be addressed.

1990; Düzgünes et al., 1992; Ramalho-Santos et al., 1993), vesicular stomatitis virus (Blumenthal et al., 1987; Clague et al., 1990) and human immunodeficiency virus 1 (Larsen et al., 1993). In order to study the mechanism of fusion, a mass-action kinetic model has been proposed for Sendai virus (Nir et al., 1986a,b) and has also been used successfully for influenza virus (Nir et al., 1986c, 1990; Stegmann et al., 1989; Düzgünes et al., 1992). The overall fusion process is analysed as a sequence of kinetically coupled reactions (Nir et al., 1983) of adhesion, dissociation and the fusion reaction *per se*. If disaggregation can be ignored and if fusion occurs immediately after adhesion of the viral particle, then adhesion will be the rate-limiting step and the overall process can be represented by a single exponential equation (Nir et al., 1986a).

In the present study we have fitted our dequenching curves to the single exponential curve but, to challenge the model, also to the sum of n exponential terms and to other models previously proposed in the literature (Wunderli-Allenspach et al., 1993). We did not use simulation curves but rather direct computer fitting of the dequencing curves to the mathematical equations. The computer program (see the Experimental section) also statistically analysed the goodness of different fits and compared the results of each fit. Finally, the choice of the best fit to different models was based on the χ^2 test and the F test of Snedecor (Lindgren, 1976). In our system, with NDV and erythocyte ghosts, we obtained the best fit with the sum of two exponential equations, thus demonstrating the existence of two independent but simultaneous processes. The first exponential term represents the viral protein-dependent fusion process itself, which follows the model of Nir et al. (1986a) and is affected by the assay conditions. The second exponential term represents a non-specific reaction, independent of the viral protein, which is also independent of the assay conditions. An interpretation of this second process is discussed in terms of an unspecific probe transfer between vesicles.

EXPERIMENTAL

Reagents

R_{18} was a product from Molecular Probes Inc. (Junction City, OR, U.S.A.). Tris, Triton X-100, BSA, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid, 4-methylumbelliferone, SDS, dithiothreitol, 2-mercaptoethanol and glycine were from Sigma (St. Louis, MO, U.S.A.). Sephadex G-75 was from Pharmacia–LKB, Uppsala, Sweden. *N*-Acetylneuraminic acid (NeuAc) and 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en) were from Boehringer, Mannheim, Germany.

Growth and purification of virus

The lentogenic strain 'Clone-30' of NDV was grown at 37 °C for 48 h in the allantoic cavity of 11-day-old specific pathogen-free chick embryos. The allantoic fluid was harvested and the virus was pelleted at 12000 *g* for 150 min in a fixed-angle rotor at 4 °C (García-Sastre et al., 1989). The resulting pellet was resuspended in 10 mM Tris/HCl buffer, pH 7.4, containing 100 mM NaCl and 1 mM EDTA, aided by a gentle 45 s sonication in a Branson B-30 sonicator. The viral suspension was then layered on to a continuous 10–50% (w/v) potassium tartrate gradient (in the same Tris/HCl buffer) and centrifuged at 80000 *g* at 4 °C for 8 h in an IEC SB-110 swinging-bucket rotor (García-Sastre et al., 1989, 1990). The viral band was collected and stored at −80 °C until used. The concentration of virus given in the text refers to the viral protein concentration.

Preparation of erythrocyte ghosts

Pig (*Sus scrofa* L. var *domestica*) blood was obtained from a local slaughterhouse and collected in a vessel containing 0.15 M NaCl/1.5% (w/v) EDTA as anticoagulant. Ghosts were prepared by hypotonic lysis of the erythrocytes in 5 mM sodium phosphate, pH 8.0, at 4 °C (Steck and Kant, 1974). Ghosts were resealed by warming in 40 vol. of 5 mM sodium phosphate, pH 8.0, containing 0.15 mM NaCl for 40 min at 37 °C. The concentration of ghosts was expressed as μg of total protein.

Preparation of R_{18}-labelled NDV membranes

The fluorescent probe R_{18} was inserted into the viral envelope essentially as described elsewhere (Hoekstra et al., 1984), with slight modifications. Briefly, 10 μl of a fresh ethanolic solution containing 300 nM probe R_{18} was injected, with vortexing, into 1 ml of KNP buffer (120 mM KCl, 30 mM NaCl and 10 mM sodium phosphate, pH 7.4) containing 5 mg of NDV protein. The mixture was incubated in the dark for 1 h at room temperature and gentle sonication was performed for 30 s every 20 min (in a Branson B-30 sonicator) in order to increase the incorporation of the probe into the viral envelope. Non-inserted probe was removed by gel-filtration chromatography on a Sephadex G-75 column (1 cm × 25 cm), with KNP as elution buffer. R_{18}-labelled virus was recovered in the void volume fraction. The total amount of fluorescent probe incorporated into the viral membrane was quantified (Hoekstra et al., 1984) by lysing the virus in 1% (v/v) Triton X-100, measuring the fluorescence and comparing this measurement with a standard curve prepared from known amounts of fluorophore solubilized in Triton X-100.

Fusion of R_{18}-labelled NDV with erythrocyte ghosts

The fusion assay is based (Hoekstra et al., 1984; Hoekstra, 1991) on fluorescence dequenching (FDQ) of the membrane-incorporated fluorophore R_{18} on fusion with eryhrocyte ghosts devoid of the probe. Continuous monitoring of the R_{18} fluorescence was carried out with a Hitachi F-4010 spectrofluorimeter, at 560 nm excitation and 590 nm emission wavelengths and with a 0.5 s time resolution. All the components within the cuvette were stirred with a magnetic stirrer during the reaction time and temperature was controlled by a thermostatically controlled circulating-water bath. To allow virus–ghost fusion, 25 μg of R_{18}-labelled NDV (in a volume of 5–15 μl of KNP buffer, pH 7.4) was added to KNP buffer and the mixture was allowed to equilibrate at the desired temperature, 37 °C. The fluorescence of the mixture was taken as zero. Subsequently, 130 μg of erythrocyte ghosts (in 5 mM sodium phosphate/0.15 mM NaCl, pH 8.0) was added and the increase in fluorescence measured for 60 min. The final volume of the reaction mixture was 2 ml. All ghost concentrations given in the text are expressed as μg of protein per total volume of the fusion mixture (2 ml). After 60 min, the fusion was stopped by the addition of Triton X-100 to a concentration of 1% (v/v) and the resulting fluorescence was taken at 100%.

To normalize the data, the percentage of fluorescence dequenching (% FDQ) at any time was calculated according to the formula:

$$\%\mathrm{FDQ}_t = \frac{F_t - F_0}{F_{100} - F_0} \times 100 \quad (1)$$

where F_0 and F_t are the fluorescence intensities at time zero and at the given time point respectively (Morris et al., 1989). F_{100}

represents the fluorescence after the disruption of the membranes by Triton X-100, which results in essentially infinite dilution of the probe.

When the effect of pH on fusion was examined, different buffers were used: Tris/HCl buffer for pH values between 4.0 and 7.0, KNP buffer for values between 7.4 and 8.0 and $NaHCO_3$ buffer for values between 9.0 and 10.0.

When the fusion of NDV with erythrocyte ghosts was studied in the presence of inhibitors of viral neuraminidase such as NeuAc and Neu5Ac2en, the inhibitor was present during the fusion reaction in the cuvette at a concentration of 50 mM for NeuAc and 0.1 mM for Neu5Ac2en, both far above the corresponding K_i (García-Sastre et al., 1991) in a final volume of 2 ml of KNP buffer, pH 7.4. The final extent of fusion was assessed by reading the fluorescence after 24 h of incubation at 37 °C of erythrocyte ghosts with R_{18}-labelled virus.

For experiments to study the fusogenic activity of NDV previously treated with reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol, 25 μg of viral protein was incubated at 37 °C in 200 μl of KNP buffer containing the appropriate concentrations of the reducing agent (2 mM DTT or 10 mM 2-mercaptoethanol). After 15 min, prewarmed buffer and erythrocyte ghosts were added up to a volume of 2 ml and the mixture was immediately transferred to a cuvette for fluorescence measurements for 60 min.

Data analysis

Fusion kinetics can be analysed by using a mass-action kinetic model (Nir et al., 1986a,b; Nir, 1991). Fusion is described by a sequence of second-order processes of virus–cell adhesion followed by the first-order fusion reaction itself. The analytical solution obtained can be expressed as the fraction [F(t)] of fused virus particles at time t (Nir et al., 1986a,b):

$$F(t) = 1-\exp(-CG_0t) \quad (2)$$

To perform our data analysis, we considered that $\%FDQ_t$ was equal to $F(t)$. Also, as the concentration of ghost particles (G_0) was kept constant in the experiments, the term CG_0 of eqn. (2) can be simplified by another constant, k. In this case, the data can be fitted to the following exponential equation:

$$\%FDQ = A[1-\exp(-kt)] \quad (3)$$

in which A is the maximum value (the asymptotic value) of %FDQ and k the constant that determines the slope of the ascending part of the curve.

Kinetic data were fitted by non-linear regression with the 'SIMFIT' computer package version 3.1 (for simulation, curve-fitting and statistical analysis, developed by W. G. Bardsley, University of Manchester, U.K.) to different models: a simple exponential equation (Nir et al., 1986a) as expressed in (3), or the sum of exponential terms:

$$\%FDQ = A_1[1-\exp(-k_1t)]+A_2[1-\exp(-k_2t)] + A_3[1-\exp(-k_3t)]+\ldots \quad (4)$$

Curve fitting was by weighted non-linear least-squares regression. This approach takes as the best estimate of calculated parameters that value of the parameter minimizing the weighted sum of squares (WSSQ):

$$WSSQ = \Sigma(y_i-y_i^*)^2/S_i^2$$

were S_i is the standard deviation of experimental errors, y_i are the experimental values and y_i^* are the values predicted for the model. In this case, WSSQ would be a χ^2 variable, and a fit could be rejected if P, the probability of the χ^2 variable exceeding WSSQ [i.e. $P(\chi^2 \geqslant WSSQ)$], is less than 0.01 (1 %).

Apart from this χ^2 test, the goodness of each individual fit was also evaluated using the run and sign tests of residuals, plots of residuals, magnitude of relative residuals, the t test for parameter redundancy and the R^2 coefficient. R^2 represents the percentage variability of the data that is explained for the mathematical model, and its values are between 0 and 1. The greater the value of R^2, the better the fit.

The choice between fits to different models was based on their respective values of χ^2 and R^2 and, basically, on the F test of Snedecor as described by Lindgren (1976). The experimental value of F was:

$$F_{exp.} = \frac{(WSSQ_1-WSSQ_2)/(p_2-p_1)}{WSSQ_2/(n-p_2)}$$

where WSSQ is the weighted sum of squared residuals in model 1 or 2, p is the number of calculated parameters for each model and n is the number of experimental data points. This experimental value ($F_{exp.}$) is F distributed with p_2-p_1 and $n-p_2$ degrees of freedom, and if $F_{exp.} > F_\alpha[(p_2-p_1), (n-p_2)]$, there is statistical support for the extra parameters and model 2 is accepted over model 1 at an α significance level.

After the best model had been chosen, the initial fusion rates were determined from the slope of the steepest part of the fitted curve, i.e. from its first derivative at time zero (Hoekstra et al., 1985).

Other procedures

Neuraminidase activity (EC 3.2.1.18) was determined by the fluorimetric procedure of Warner and O'Brien (1979), essentially as described by Cabezas et al. (1985), using 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid as substrate. Protein measurements were performed by the spectrophotometric procedure of Markwell et al. (1978) for membrane proteins, using BSA as standard.

RESULTS

Virus labelling

The fluorescent probe R_{18} is readily incorporated into the viral membrane after being injected in an ethanolic solution into the virus-containing medium. As the lipid/protein ratio in the NDV membrane is around 0.2 (I. Muñoz-Barroso and E. Villar, unpublished work), the surface density of the probe should be around 5–10 mol % with respect to the total viral lipid. Under these conditions, a self-quenching of 60–90 % is obtained, which allows continuous monitoring of the fusion process. To exclude the possibility that virus labelling might affect the other membrane-bound protein, HN, which has haemagglutinin and neuraminidase activities, neuraminidase activity was tested and compared with that of unlabelled virus. No differences in neuraminidase activity were found in labelled and unlabelled viruses, even when labelling was higher than 10 mol % with respect to the total viral lipid content (results not shown). Controls were carried out to exclude the possible interference of the probe in the neuraminidase assay (results not shown).

Kinetics of virus–ghost fusion

All fluorescence data obtained were transformed to %FDQ versus time, and the transformed data fitted with the 'SIMFIT' computer package to different exponential models, as described

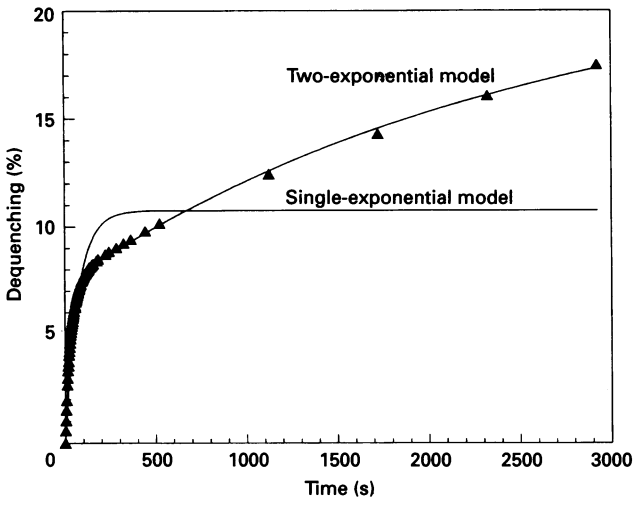

Figure 1 Comparison between one-exponential and two-exponential computer fits of the R_{18} dequenching curves

R_{18}-labelled NDV (25 μg of protein) was incubated in the presence of 130 μg of ghost protein, in a total volume of 2 ml of KNP buffer, pH 7.4, at 37 °C for 60 min. FDQ curves were normalized and computer-fitted to the corresponding mathematical equation (see the Experimental section). Experimental values are indicated by data points and fitted curves by the drawn lines.

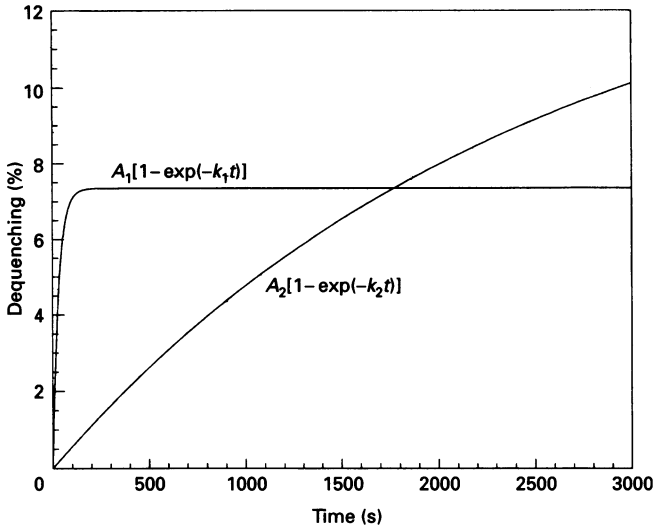

Figure 2 Representation of each exponential equation resulting from the two-exponential R_{18} dequenching curve

Both equations follow the equation $\%FDQ_t = A[1-\exp(kt)]$. For the first exponential, representing the viral protein-specific fusion reaction, constant values are: $A_1 = 7.35$ and $k_1 = 321.2 \times 10^{-4}$ s^{-1}. For the second exponential, corresponding to a non-specific process, the values are: $A_2 = 14.54$ and $k_2 = 3.97 \times 10^{-4}$ s^{-1}.

above. For a single-exponential model (Nir et al., 1986a), a value of 0.8 was found for the coefficient R^2, and with the χ^2 test the value of $P = 0.00$. However, when data were fitted to the sum of two exponentials [eqn. (4)], the value was 1 for both R^2 and P, indicating a much better fit. Fitting to the sum of a higher order of exponentials did not increase the goodness of fit, there being a lack of statistical support for additional parameters. The difference between fits to one exponential and the sum of two exponentials is shown in Figure 1. Using the F test (Lindgren, 1976) the single-exponential model had to be rejected and the two-exponential model accepted at the 0.1 % significance level.

The fitting of our data to the sum of two exponentials allows us to split the FDQ curve into two different but simultaneous reactions:

$$\%FDQ = A_1[1-\exp(-k_1t)] + A_2[1-\exp(-k_2t)] \quad (5)$$

The first exponential term in eqn. (5) represents a fast dequenching reaction, with high values of k_1 (which determines the slope of the ascending part of the curve) reaching the maximum value, A_1, very quickly; however, the asymptotic value, A_1, is small with respect to the final dequenching. The second exponential term represents a slow dequenching reaction, with a smaller slope value, k_2, but with a higher asymptotic

Table 1 Kinetic constants of the NDV–ghost fusion R_{18} dequenching curves: influence of pH and temperature

A control experiment was carried out with 25 μg of R_{18}-labelled NDV protein and 130 μg of erythrocyte ghost protein in a total volume of 2 ml of KNP buffer, pH 7.4, at 37 °C. Only pH or temperature was changed in each assay. Kinetic parameters were obtained by fitting the normalized dequenching curves (see the Experimental section) to the sum of two exponentials. Fitted parameters are listed with their 95% confidence limits. Initial rates were calculated as the slope of the dequenching curves at time zero.

		First exponential		Second exponential	
	$10^{-2} \times$ Initial fusion rate (%/s)	A_1	$K_1 \times 10^4$ (s^{-1})	A_2	$K_2 \times 10^4$ (s^{-1})
pH					
4.0	1.2	1.9±0.3	31.1±4.1	14.8±0.4	4.1±0.4
5.0	2.6	3.1±0.1	59.8±3.2	18.8±0.5	3.9±0.2
6.0	8.5	5.3±0.1	146.1±2.9	20.4±0.6	3.3±0.2
7.4	24.0	7.3±0.1	321.2±8.8	14.5±1.3	4.0±0.6
8.0	12.0	5.8±0.2	197.7±13.3	12.6±1.3	4.2±0.9
9.0	7.1	4.3±0.1	137.8±8.2	18.1±0.3	6.1±0.3
10.0	1.1	0.9±0.2	49.5±9.2	14.7±0.3	4.3±0.3
Temperature (°C)					
10	0.2	0.2±0.1	46.7±11.5	10.1±1.1	1.4±0.2
20	0.4	1.1±0.3	17.6±2.7	15.6±3.9	1.2±0.4
30	9.1	6.4±0.2	134.0±8.0	12.4±0.8	4.6±0.7
37	24.0	7.3±0.1	321.2±8.8	14.5±8.8	4.0±0.6

Table 2 Kinetic constants of the NDV–ghost fusion R_{18} dequenching curves: influence of ghost concentration

All assays were carried out with 25 μg of R_{18}-labelled NDV proteins and the indicated amount of erythrocyte ghost protein in a total volume of 2 ml of KNP buffer, pH 7.4, at 37 °C. Kinetic parameters were obtained by fitting the normalized dequenching curves (see the Experimental section) to the sum of two exponentials. Fitted parameters are listed with their 95% confidence limits. Initial rates were calculated as the slope of the dequenching curves at times zero.

Ghost concn. (μg)	10^{-2} × Initial fusion rate (%/s)	First exponential		Second exponential	
		A_1	$k_1 \times 10^4$ (s^{-1})	A_2	$k_2 \times 10^4$ (s^{-1})
17	4.9	2.1 ± 0.03	216.6 ± 8.4	13.7 ± 1.3	1.8 ± 0.2
34	8.1	3.3 ± 0.03	234.6 ± 6.4	12.8 ± 0.5	2.7 ± 0.2
68	16.0	6.2 ± 0.05	244.6 ± 6.4	12.6 ± 0.8	3.1 ± 0.3
130	24.0	7.3 ± 0.10	321.2 ± 8.8	14.5 ± 1.3	4.0 ± 0.6
250	26.0	9.1 ± 0.30	281.4 ± 24.6	13.5 ± 0.9	6.4 ± 1.1
400	25.0	9.7 ± 0.4	253.7 ± 30.3	11.3 ± 0.9	7.8 ± 2.0

Table 3 Kinetic constants of the NDV–ghost fusion R_{18} dequenching curves in the presence of disulphide-reducing agents and viral neuraminidase inhibitors

R_{18}-labelled NDV proteins (25 μg) were incubated in the presence (2 mM DTT or 10 mM 2-mercaptoethanol) of each reducing agent in 200 μl of KNP buffer, pH 7.4, at 37 °C for 15 min. Then, 130 μg of ghost proteins and KNP buffer were added up to 2 ml and R_{18} FDQ was monitored. Where indicated, inhibitors (50 mM NeuAc or 0.1 mM Neu5Ac2en) were present during the fusion reaction, under the same conditions as the control. Kinetic parameters were obtained by fitting the normalized dequenching curves (see the Experimental section) to the sum of two exponentials. Fitted parameters are listed with their 95% confidence limits. Initial rates were calculated as the slope of the dequenching curves at time zero.

Additions	10^{-2} × Initial fusion rate (%/s)	First exponential		Second exponential	
		A_1	$k_1 \times 10^4$ (s^{-1})	A_2	$k_2 \times 10^4$ (s^{-1})
Control	24.1	7.3 ± 0.1	321.2 ± 8.8	14.5 ± 1.2	4.0 ± 0.6
DTT	2.9	3.2 ± 0.1	75.4 ± 2.4	13.1 ± 0.5	3.4 ± 0.3
2-Mercaptoethanol	0.9	2.1 ± 0.2	24.5 ± 1.6	18.4 ± 0.8	2.4 ± 0.2
Neu-Ac	1.9	4.2 ± 0.7	27.8 ± 3.1	15.9 ± 0.2	4.7 ± 0.5
Neu5Ac2en	0.5	1.1 ± 0.5	19.9 ± 6.2	22.8 ± 3.8	1.6 ± 0.5

value, A_2. Figure 2 shows the plots of both exponential terms obtained with computer-fitted data from a fusion experiment.

Influence of factors that affect the kinetics of fusion

In order to investigate the biological meaning of the two exponential terms in eqn. (5), we studied the influence on the exponential parameters A and k of factors that are well known to affect fusion in enveloped viruses: pH, temperature, concentration of erythrocyte ghosts and reducing agents. The effect of competitive inhibitors of neuraminidase was also tested.

The effect of pH was checked between 4.0 and 10.0. To start the reaction, 130 μg of ghost protein was added to the cuvette containing 25 μg of R_{18}-labelled NDV in buffer at the appropriate pH, at 37 °C. The fusion process was measured and the data were handled as described above. In accordance with other data (Lorge et al., 1986), the initial rate of fusion in NDV is pH-dependent, with a bell-shaped curve displaying a maximum at neutral pH (pH 7.4) (Table 1), as found for Sendai virus (Hoekstra et al., 1985), another paramyxovirus. The variations in the parameters A and k of both exponential terms are summarized in Table 1. For the first exponential, both parameters, A_1 and k_1, are pH-dependent with maximum values at pH 7.4 and a bell-shaped curve similar to the pH-dependent fusion curve. However, the changes found in the parameters, A_2 and k_2, of the second exponential term cannot be related to variations in pH and lie within a very narrow range.

The effect of temperature on the parameters of the two exponentials was also investigated. Fusion of NDV with erythrocyte ghosts is temperature-dependent, with a maximum at 37 °C, showing a sharp decrease at higher or lower temperatures (results not shown) in agreement with the results obtained for other paramyxoviruses (Hoekstra et al., 1985). The variation in the parameters A and k of both exponentials are summarized in Table 1. For the first, both parameters, A_1 and k_1, are also temperature-dependent having a maximum at 37 °C. However, there are only very small differences with temperature in the values of A_2 and k_2.

When the effect of the concentration of erythrocyte ghosts on fusion was studied, we found, as expected (Hoekstra et al., 1985), an increase in the initial rate of fusion with ghost concentration, up to 250 μg (Table 2). The parameter k, representing the slope of the ascending part of the first exponential, shows little variation with increasing ghost concentration. However, the value of A_1, which represents the final extent (asymptotic value) of fusion, is higher with increasing ghost concentration, up to the above-mentioned limit of 250 μg of ghosts. The changes in the parameters of the second exponential, A_2 and k_2, again cannot be related to ghost concentration.

We also explored the effect of two reducing agents, DTT and 2-mercaptoethanol, on the parameters of the two fusion exponentials. As expected (Hoekstra and Klappe, 1986), the presence of these compounds inhibited the fusion process, the initial rate of fusion being reduced by a factor of about 10 (Table 3). The parameters of the first exponential were much lower in the presence of the reducing agents, but those of the second

exponential did not change in the presence of these agents with respect to the control reaction.

The presence of the competitive inhibitors of neuraminidase activity, NeuAc and Neu5Ac2en, also produced a decrease in fusion (Table 3). The extent of inactivation depended on the inhibitory power of the compound. Neu5Ac2en, which is a very strong competitive inhibitor of NDV neuraminidase activity [K_i 27.6 μM with 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid as substrate (García-Sastre et al., 1991)], dramatically reduced fusion with respect to both the initial fusion rate and the parameters of the first exponential term (Table 3). NeuAc, which is a weak inhibitor [K_i 11.2 mM with the above-mentioned substrate (García-Sastre et al., 1991)], was required at a much higher concentration to produce only a small decrease in fusion (Table 3). Neither of the compounds affected the parameters of the second exponential equation (Table 3).

DISCUSSION

In this work we have used FDQ of the membrane probe R_{18} (Hoekstra et al., 1984) to study the fusogenic activity of NDV with erythrocyte ghosts. Computer analysis allowed us to fit the experimental data to the sum of two exponential equations [eqn. (5)], thus demonstrating the existence of two independent, but simultaneous, processes. One reaction, which we will call the first reaction (the first exponential equation from the kinetic analysis), represents FDQ due to the virus–ghost fusion in a viral-protein-dependent process, because this reaction was found to be affected by the reaction conditions (alterations in the fusion process because modifications of the assay conditions yield changes in the kinetic constants of the first reaction). The second exponential equation represents the second reaction of the process, the constants of which hardly change when the assay conditions are modified. This second reaction is therefore basically independent of the assay conditions and corresponds to a process that needs further explanation (see below).

Several authors have fitted the experimental data to other second-order functions (Wunderli-Allenspach and Ott, 1990; Wunderli-Allenspach et al., 1993). Our experimental data do fit those other models but the goodness of fit is much better for the sum of two exponentials, and indeed the only one that passes all statistical tests. However, in addition to this statistical rationale, it is necessary to consider theoretical kinetic analysis. These analyses indicate that, although empirical modelling of the data could allow fitting to different equations, in the case of virus–cell fusion (Nir et al., 1986a,b) there is one theoretical single-exponential model that will best explain the fusion process (Lyles and Landsberger, 1979; Tsao and Huang, 1986; Nir et al., 1986a,b; Pedroso de Lima et al., 1991, 1992; Ramalho-Santos et al., 1993). All of the dequenching curves obtained in the course of our investigations gave the best fit to the proposed model of the sum of two exponentials, regardless of the assay conditions (pH, temperature, ghost concentration, etc.).

The first exponential equation describes a very fast process, lasting only a short period of time, which reaches a maximum value of 7.35% of FDQ. This is a very low value, representing just one-third of the dequenching obtained after 60 min of fusion, and shows that only a small percentage of NDV is able to fuse in a specific reaction that depends on the viral proteins. This low fusion rate is not surprising, however, because the 'Clone-30' strain used in this study is lentogenic, with low pathogenicity and fusion capabilities, even being used as a veterinary vaccine.

The second exponential equation represents a reaction that is much slower than the first, with a $k_2 = 3.97 \times 10^{-4}\ s^{-1}$ and $t_{1/2} =$ 30 min. This reaction reaches a maximum of about 14% of the FDQ, representing two-thirds of the total dequenching obtained after 60 min of fusion.

To investigate further the possible differences between the two reactions and to understand their meaning, the fusion process was studied by changing the conditions known to affect it. When fusion was performed at different pHs, maximum fusogenic activity was obtained at pH 7.4 (Table 1), as previously found (Hoekstra et al., 1985; Lorge et al., 1986). Analysis of the kinetic constants of the two exponentials at different pHs (Table 1) reveals that only those of the first equation change, whereas those of the second do not show pH-dependent variation. The small variations observed for A_2 are due to the fact that the kinetic fit was performed after 60 min of fusion. This second reaction is much slower than the first and needs much more time to reach its maximum (asymptotic) value. When fusion proceeded for 24 h, no differences were found and all the values of A_2 at different pH values were about 45% of FDQ. The reasons why kinetic fitting was not performed after 24 h of fusion are: (i) it is difficult to follow the fusion process for such a long time and (ii) in the fusion curves, most of the important information is obtained after a short time, and, if fitting is performed after a very long time, the data on FDQ are overweighted at the expense of those obtained after a short time, which gives much more information, thus biasing the fit.

When fusion was followed at different temperatures (between 10 and 45 °C), the initial velocity was maximum at 37 °C (Table 1) in agreement with data published elsewhere (Hoekstra et al., 1985; Chejanovsky and Loyter, 1985; Pedroso de Lima et al., 1992). Again, the kinetic constants of the two exponential equations behaved differently, the parameters from the second exponential showing only very small increments with temperature.

The same differences in behaviour of the two exponentials can be demonstrated when fusion is performed with increasing ghost concentration (Table 2). In this case, the parameter that changes most in the first exponential equation is A_1, the dequenching asymptotic value, showing a maximum that corresponds to 11% of FDQ, thus corresponding to the maximum percentage of virions able to fuse with erythrocyte ghosts. The constant, k_1, representing the velocity of the reaction, hardly changes with ghost concentration. This is not surprising, because the conditions of the assay are not changed and the only variable is the number of target cells. The constants of the second term show only a slight increase.

Regarding the use of R_{18} as a fluorescent probe to follow virus–cell fusion, R_{18} transfer from small unilamellar vesicles to influenza virus has been reported (Wunderli-Allespach and Ott, 1990). Data on this issue are, however, contradictory because other authors did not find such a non-specific probe transfer between PC12 cells and influenza-labelled virus (Ramalho-Santos et al., 1993). However, others (Wunderli-Allespach et al., 1993) did find probe transfer and also inactivation of influenza virus HA glycoprotein and viral fusion by high concentrations of probe R_{18}. To our knowledge, there are no references in the literature to non-specific fluorescence dequenching when R_{18}-labelled NDV is assayed with other membrane systems. Our results also show that fusogenic activity is not inactivated by increasing R_{18} labelling and that the fusion-specific reaction always represents approximately one-third of the FDQ after 1 h, regardless of the R_{18} concentration in the membrane. It seems therefore that R_{18} does not affect the biological activity of NDV membrane-bound proteins. Indeed, the neuraminidase activity of NDV HN glycoprotein does not change when R_{18} is present in the viral membrane, even at the highest concentration.

From these results, we believe that the second exponential equation represents a process that is independent of the assay conditions and could represent a non-specific probe transfer between membranes, probably caused by random vesicle collisions in the absence of fusion, thus being independent of the viral proteins. The non-specific probe transfer is independent of pH and almost independent of temperature (within the temperature range assayed). This non-specific transfer could be responsible for the fact that the maximum FDQ in all the experiments, regardless of the assay conditions, was always the same after very long fusion times (24 h).

This probe transfer prevents us from determining the number of virions that can fuse with a single ghost. For the same reason, binding experiments (Hoekstra and Klappe, 1986) are difficult to perform because of this non-specific process occurring simultaneously with the specific fusion reaction. However, preliminary results (not shown) indicate that approximately 70% of the labelled viral particles can bind erythrocyte ghosts, if the latter are present at a high enough concentration. In conclusion, 10% of bound viruses are able to fuse and do so in a very short period of time. In addition, no lag phase is observed at the beginning of the fusion reaction. Thus no difference is observed in the fusion kinetics with or without prebinding (at 4 °C for 15 min), in the initial velocity or in maximum FDQ. No lag phase is observed at low temperatures (between 10 and 20 °C) either, and hence binding seems to be the rate-limiting step.

We have also assayed the effect on fusion kinetics of chemical compounds known to interfere with the biological activities of NDV membrane-associated proteins, namely disulphide reducing agents and viral neuraminidase competitive inhibitors.

F protein is composed of two disulphide-linked subunits, F_1 and F_2, and maintenance of these disulphide bridges is essential for fusion to take place (Hoekstra et al., 1985). In our experiments, the initial velocity decreased in the presence of the disulphide-reducing compounds (Table 3), in accordance with results obtained with Sendai virus (Hoekstra et al., 1985; Hoekstra and Klappe, 1986). Again, our results show that the fusion kinetics fit the sum of two exponentials and also that the first equation is the only one in which the constants are modified.

In the presence of competitive inhibitors of viral neuraminidase (NeuAc and Neu5Ac2en), the initial fusion velocity is decreased (Table 3). Both the velocity (k_1) and the fusion maximum (A_1) of the first exponential equation are lower in the presence of the inhibitors, but to different extents (Table 3) depending on the inhibition constant of the compound. The kinetic constants of the second exponential equation, reflecting non-specific probe transfer, hardly change in the presence of these inhibitors. These results again demonstrate that factors affecting the biological activity of NDV membrane-bound glycoproteins affect the fusion process only in its first exponential term, which represents the specific fusion reaction and not the proposed non-specific probe transfer. These results, demonstrating that fusion is reduced when neuraminidase activity is decreased by inhibitors, support the findings of other authors who have proposed that the NH glycoprotein is necessary for the fusion process to occur (Miura et al., 1982; Citovski and Loyter, 1985; Citovski et al., 1986; Morrison et al., 1991; Moscona and Peluso, 1991; Iorio et al., 1992). However, the possibility that the inhibitors used here might also interact with the F protein cannot be excluded, although this would be unlikely as the HN protein is the only known viral sialic acid-containing receptor-binding protein.

The reason for this non-specific probe transfer during the R_{18}-labelled NDV–cell fusion process is unclear. To our knowledge, it has not been previously detected in other paramyxoviruses. We have also checked the possibility of probe transfer between ghosts by incubating R_{18}-labelled ghosts (with an initial quenching of 70%) with unlabelled ghosts, under the assay conditions (37 °C, pH 7.4); the observed %FDQ was very small, reaching no higher than 6% after 1 h. This means that a certain amount of probe transfer does occur between ghosts, but it is too small to explain the transfer rate found in fusion assays. However, when R_{18}-labelled NDV virions are incubated under the same conditions in the presence of unlabelled virions at a 1:1 ratio, 20% of FDQ is found after 1 h. This FDQ also fits a double-exponential model. The first exponential term, with a velocity constant similar to that of the fusion between ghosts, represented only one-fifth of the FDQ, and thus seems to be due to a small amount of fusion between virions, as previously described for 'aged' virions (Kim and Okada, 1987). However, the second exponential term accounts for most of the observed FDQ. In conclusion, there is active R_{18} transfer between virions and between virions and ghosts, and a very small amount of transfer between ghosts. It has been suggested (Hoekstra et al., 1984) that the stability of the probe in the bilayer could depend not only on its acyl chain length but also on the interactions that its head can establish with the phospholipids in the bilayer. Because the lipid composition of the NDV bilayer is different (I. Muñoz-Barroso and E. Villar, unpublished work) from that of other viruses used in fusion assays, it is possible that the probe could be less stable in such a membrane, thus facilitating probe transfer.

In conclusion, using detailed computer-assisted fitting, we have shown that, under our conditions, the fusion process of R_{18}-labelled NDV with erythrocyte ghosts proceeds via two different but simultaneous processes: one is specific to the viral membrane-associated proteins, mainly the F protein, and the other is due to a non-specific probe transfer between membranes. Thus, although non-specific probe transfer does occur, it is possible to characterize it and therefore to obtain true data for the specific fusion. The fluorescent method using R_{18}-labelled virus (Hoekstra et al., 1984) therefore seems to be excellent for monitoring virus–cell fusion, although special care is needed in handling the dequenching curves; the existence of the non-specific probe transfer must be considered and the data should be fitted to appropriate equations in order to distinguish the true fusion data from the probe transfer. We also conclude, with strong statistical support, that the kinetic model previously proposed by Nir et al. (1986a,b) is the best one for describing the NDV–erythrocyte ghost fusion process, once the probe transfer has been eliminated.

We are particularly indebted to Dr. W. G. Bardsley (University of Manchester) for his gift of the computer program 'SIMFIT' developed by him. We thank E. Diez de Espada and J. A. Rodriguez Hernández, from Laboratories Intervet, Salamanca, Spain, for providing the lentogenic 'Clone-30' strain. We are also grateful to Dr. F. J. Burguillo (Department of Physical Chemistry, University of Salamanca, Spain) for his helpful suggestions and to Professor J. A. Cabezas (Department of Biochemistry and Molecular Biology, University of Salamanca, Spain) for his valuable discussions. C.C. is a fellowship holder of the Plan de Formación del Profesorado Universitario y Personal Investigador, Ministerio de Educación y Ciencia, Spain. E. V. is the recipient of an Acciones Concertadas grant from the University of Salamanca. Thanks are also due to Mr. N. S. D. Skinner for proof-reading the manuscript.

REFERENCES

Blumenthal, R., Bali-Puri, A., Walter, A., Covell, D. and Eidelman, O. (1987) J. Biol. Chem. **262**, 13614–13619

Cabezas, J. A., Calvo, P., Eid, P., Martin, J., Perez, N., Reglero, A., Rodrigo, M. and Hannoun, C. (1985) Int. J. Biochem. **14**, 311–319

Chejanovsky, N. and Loyter, A. (1985) J. Biol. Chem. **260**, 7911–7918

Choppin, P. W. and Compans, R. W. (1975) Compr. Virol. **4**, 95–178

Choppin, P. W. and Scheid, A. (1980) Rev. Infect. Dis. **2**, 40–61

Citovski, V. and Loyter, A. J. (1985) J. Biol. Chem. **260**, 12072–12077

Citovski, V., Yanai, P. and Loyter, A. J. (1986) J. Biol. Chem. **261**, 2235–2239

Clague, M. J., Schoch, C., Zech, L. and Blumenthal, R. (1990) Biochemistry **29**, 1303–1308
Düzgünes, N., Pedroso de Lima, M. C., Stamatatos, L., Flasher, D., Alford, D., Friend, D. S. and Nir, S. (1992) J. Gen. Virol. **73**, 27–37
Galinski, M. S. and Wechsler, S. L. (1991) in The Paramyxoviruses (Kingsbury, D. W., ed.), pp. 41–82, Plenum Press, New York
García-Sastre, A. Cabezas, J. A. and Villar, E. (1989) Biochim. Biophys. Acta **999**, 171–175
García-Sastre, A., Corral, J., Cabezas, J. A. and Villar, E. (1990) Biochem. Educ. **18**, 197–200
García-Sastre, A., Cobaleda, C., Cabezas, J. A. and Villar, E. (1991) Biol. Chem. Hoppe-Seyler **372**, 923–927
Hoekstra, D. (1991) in Membrane Fusion (Wilschut, J. and Hoekstra, D., eds.), pp. 289–312, Marcel Dekker Inc., New York
Hoekstra,. D. and Klappe, K. (1986) J. Virol. **58**, 87–95
Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) Biochemistry **23**, 5675–5681
Hoekstra, D., Klappe, K., De Boer, T. and Wilschut, J. (1985) Biochemistry **24**, 4739–4745
Hoekstra, D., Klappe, K., Hoff, H. and Nir, S. (1989) J. Biol. Chem. **264**, 6786–6792
Iorio, R. M., Glickman, R. L. and Sheehan, J. P. (1992) J. Gen. Virol. **73**, 1167–1176
Kim, J. and Okada, Y. (1987) J. Membr. Biol. **97**, 241–249
Klappe, K., Wilschut, J., Nir, S. and Hoekstra, D. (1986) Biochemistry **25**, 8252–8260
Larsen, C. E., Nir, S., Alford, D. R., Jennings, M., Lee, K. D. and Düzgünes, N. (1993) Biochim. Biophys. Acta **1147**, 223–236
Lindgren, B. W. (1976) in Statistical Theory, pp. 542–545, MacMillan, New York
Lorge, P., Cabiaux, V., Lê Long and Ruysschaert, J. M. (1986) Biochim. Biophys. Acta **858**, 312–316
Lyles, D. S. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 5621–5625
Lyles, D. S. and Landsberger, F. R. (1979) Biochemistry **18**, 5088–5095
Markwell, M. A. K., Haas, S. M., Bieber, L. L. and Tolbert, N. N. (1978) Anal. Biochem. **87**, 200–210
Miura, N., Uchida, T. and Okada, Y. (1982) Exp. Cell. Res. **141**, 409–420
Morris, S. J., Sarkar, D. P., White, J. M. and Blumenthal, R. (1989) J. Biol. Chem. **264**, 3972–3978
Morrison, T. (1988) Virus Res. **10**, 113–136
Morrison, T. and Portner, A. (1991) in The Paramyxoviruses (Kingsbury, D. W., ed.), pp. 347–382, Plenum Press, New York
Morrison, T., McQuain, C. and McGuinnes, L. W. (1991) J. Virol. **65**, 813–822
Moscona, A. and Peluso, R. W. (1991) J. Virol. **65**, 2773–2777
Nir, S. (1991) in Membrane Fusion (Wilschut, J. and Hoekstra, D., eds.), pp. 127–154, Marcel Dekker, New York
Nir, S., Bentz, J., Wilschut, J. and Düzgünes, N. (1983) Progr. Surf. Sci. **13**, 1–124
Nir, S., Klappe, K. and Hoekstra, D. (1986a) Biochemistry **25**, 2155–2161
Nir, S., Klappe, K. and Hoekstra, D. (1986b) Biochemistry **25**, 8261–8266
Nir, S., Stegmann, T. and Wilschut, J. (1986c) Biochemistry **25**, 257–266
Nir, S., Düzgünes, N., Pedroso de Lima, M. C. and Hoekstra, D. (1990) Cell Biophys. **17**, 181–201
Pedroso de Lima, M. C., Nir, S., Flasher, D., Klappe, K., Hoekstra, D. and Düzgünes, N. (1991) Biochim. Biophys. Acta **1070**, 446–454
Pedroso de Lima, M. C., Ramalho-Santos, J., Martins, M. F., Pato de Carvalho, A., Bairos, V. and Nir, S. (1992) Eur. J. Biochem. **205**, 181–186
Ramalho-Santos, J., Nir, S., Düzgünes, N., Pato de Carvalho, A. and Pedroso de Lima, M. C. (1993) Biochemistry **32**, 2771–2779
Rett, R. (1979) Arch. Virol. **59**, 285–298
Steck, T. L. and Kant, J. A. (1974) Methods Enzymol. **31**, 172–180
Stegmann, T., Nir, S. and Wilschut, J. (1989) Biochemistry **28**, 1698–1704
Tsao, Y. and Huang, L. (1986) Biochemistry **25**, 3971–3976
Warner, T. G. and O'Brien, J. S. (1979) Biochemistry **18**, 2783–2787
White, J. M. (1990) Annu. Rev. Physiol. **52**, 675–697
White, J. M. (1992) Science **258**, 917–924
Wunderli-Allenspach, H. and Ott, S. (1990) Biochemistry **29**, 1990–1997
Wunderli-Allenspach, H., Günthert, M. and Ott, S. (1990) J. Struct. Biol. **104**, 63–69
Wunderli-Allenspach, H., Günthert, M. and Ott, S. (1993) Biochemistry **32**, 900–907

Received 29 September 1993/17 December 1993; accepted 31 December 1993