Components involved in virally mediated membrane fusion and permeability changes

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1. Intact F glycoprotein is required to induce permeability changes in Lettrée cells or in erythrocytes. Some HN glycoprotein may also be required. Permeability changes thus offer a simple, accurate and rapid means of assaying the integrity of F glycoprotein in certain viral preparations. 2. The '1-day' virus (which contains intact F glycoprotein but which differs morphologically from '3-day' virus) does not cause permeability changes; it can be rendered active by various physical treatments. It is concluded that the environment in which F glycoprotein is embedded is a determining factor for permeability changes. 3. The entry of fluorescently labelled peptides into cells made permeable by virus has been measured. Peptides having a molecular weight in excess of 1000 enter poorly, suggesting a 'pore' size of approx. 1 nm in diameter. 4. Two novel assay methods concerned with virus–cell fusion are described. The first measures the fluorescence enhancement that occurs when anthroylstearate is transferred from anthroylstearate-labelled virus to cells. The second measures the giant-cell formation that occurs when partially fused erythrocytes are exposed to hypo-osmotic treatment. The '1-day' virus is active in these assays. In contrast with permeability changes, virus–cell fusion is insensitive to changes in external Ca^{2+} concentration. 5. The results are compatible with a model [Knutton & Pasternak (1979) *Trends Biochem. Sci.* **4**, 220–223; Impraim, Foster, Micklem & Pasternak (1980) *Biochem. J.* **186**, 847–860] in which virus–cell fusion is a prerequisite for permeability changes, and in which permeability changes are the cause of haemolysis and giant-cell (polykaryon) formation.

We have suggested that permeability changes caused by certain paramyxoviruses are (a) the consequence of virus–cell fusion and (b) the cause of haemolysis and giant-cell (polykaryon) formation (Knutton & Pasternak, 1979; Impraim *et al.*, 1980). Of the two glycoproteins present in the envelope of these paramyxoviruses, termed F and HN (Mountcastle *et al.*, 1971), F glycoprotein has been implicated in virus–cell fusion (Maeda *et al.*, 1977), in haemolysis (Hsu *et al.*, 1979) and in cell–cell fusion (Homma & Ohuchi, 1973; Scheid & Choppin, 1974; Hosaka & Shimizu, 1977). [F glycoprotein is in fact an oligomer made up of two subunits (F_1 and F_2) joined by a disulphide bond (Scheid & Choppin, 1974).] If our hypothesis is correct, it is to be expected that F glycoprotein is a necessary factor for virally mediated permeability changes also. Moreover '1-day' virus (Homma *et al.*, 1976), which is infective but does not cause haemolysis, should not cause permeability changes. Here we confirm both these predictions, and make

an estimate of the size of pore through which increased permeability takes place.

Another of our postulates is that virus–cell fusion, in contrast with subsequent permeability changes leading to haemolysis and giant-cell formation, is not affected by Ca^{2+} (Knutton & Pasternak, 1979; Impraim *et al.*, 1979, 1980). At present few convenient methods for assay of F glycoprotein or virus–cell fusion exist. In the present work we have developed two assay methods and have shown that, in each, activity is unaffected by concentrations of Ca^{2+} that inhibit permeability changes.

Methods and materials

Mouse-grown Lettrée cells and Sendai virus were prepared as described in Impraim *et al.* (1980); unless stated otherwise, virus was of the '3-day' type. For some experiments virus was purified by zonal centrifugation at 100 000 *g* through 0–55% (w/v) sucrose for 150 min at 4°C. The opalescent virus-

containing fractions (at approx. 39% sucrose) were dialysed against Hepes-buffered saline [150 mM-NaCl/5 mM-KCl/5 mM-4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid/NaOH buffer, pH 7.2] and concentrated by pelleting at 21 000 *g* for 20 min at 4°C. Unless stated otherwise, unpurified virus was used throughout. Anti-(Sendai virus) serum was obtained from rabbits injected, three times, at weekly intervals with 5000 haemagglutination units of purified virus in complete Freund's adjuvant. Heated virus refers to virus heated in a boiling-water bath for 2 min.

Iodination of virus was by the method of Fraker & Speck (1978), with 500 μ Ci of Na¹²⁵I (The Radiochemical Centre, Amersham, Bucks., U.K.) and 1 ml of purified virus suspension (3 mg/ml); excess ¹²⁵I was removed by pelleting the virus and washing three times with Hepes-buffered saline.

Reassembly of viral envelope components was carried out by stirring purified virus (3 mg/ml) in the presence of 0.25% Nonidet NP 40 (BDH Chemicals, Poole, Dorset, U.K.) and phenylmethane-sulphonyl fluoride (0.1 mM) at 22°C for 15 min, centrifuging at 100 000 *g* for 40 min at 4°C and dialysing the supernatant against SM2 beads (Bio-Rad Laboratories) suspended in Hepes-buffered saline for 4 h at 22°C, followed by 2–3 days at 4°C, Spectrapor 2 dialysis tubing (Raven Scientific, Haverhill, Suffolk, U.K.) being used. Alternatively, for the experiment described in Table 1, viral envelope components were solubilized in Triton X-100 (Sigma Chemical Co., Poole, Dorset, U.K.) by the method of Volsky & Loyter (1978a).

Proteolytic degradation of virus by trypsin (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) or *Staphylococcus aureus* V8 proteinase (Miles Laboratories, Stoke Poges, Slough, Berks., U.K.) was carried out by incubating virus (3 mg/ml) with enzyme at various concentrations for 1 h at 35°C, unless stated otherwise. The preparation was chilled and washed with Hepes-buffered saline by centrifugation at 21 000 *g* for 20 min at 5°C.

Haemagglutination activity (expressed as haemagglutination units) of virus was determined by the method of Martin (1978). Haemolysis activity was determined by adding virus to a 1% suspension of human or sheep erythrocytes in Hepes-buffered saline, incubating for 15 min at 35°C, centrifuging and assessing the absorbancy of the supernatant at 560 nm. Leakage was determined by incubating virus with Lettrée cells (2×10^7 /ml), previously exposed to [³H]choline (The Radiochemical Centre) for 30 min at 37°C and washed, for 15 min at 35°C and measuring ³H released as previously described (Pasternak & Micklem, 1973, 1974a). Uptake was determined by spinning through oil as described by Impraim *et al.* (1979). Binding of ¹²⁵I-labelled virus was determined by incubating virus (100 haemag-

glutination units) with Lettrée cells (8×10^6) in a final volume of 0.7 ml at 4°C or 35°C for 15 min, washing three times in ice-cold Hepes-buffered saline and measuring ¹²⁵I in the pellet.

Fluorescently labelled virus was prepared by incubating 3 mg of purified virus with 0.25 mg of DL-12-(9-anthroyl)stearic acid (Sigma Chemical Co.) in 1 ml of Hepes-buffered saline for 2 min at 22°C with shaking, and washing twice with Hepes-buffered saline. For fluorescence enhancement assay, anthroylstearate-labelled virus (100 haemagglutination units) was incubated with Lettrée cells (8×10^6) in a final volume of 0.7 ml at 35°C, or other temperature as stated, for 10–20 min, and fluorescence intensity was measured at 440 nm in a Perkin-Elmer MPF44A fluorimeter with a xenon lamp (excitation at 366 nm); the fluorimeter was operated in the a.c. ratio mode. The amount of anthroylstearate transferred to Lettrée cells was determined by washing cells three times at the end of 20 min of incubation and measuring the fluorescence intensity of the cell pellet; although this includes any virus bound to, but not fused with, cells, the intensity in the absence of appreciable enhancement was negligible. In every case, the fluorescence intensity of the cell pellet was approx. 95% of that of the cell-virus suspension at the end of the 20 min incubation time.

Optical assay of virus-cell fusion was carried out by adding virus (500–1000 haemagglutination units/ml) to monolayers of human erythrocytes adsorbed on poly-L-lysine-coated plastic Petri dishes as described by Bachi *et al.* (1978). The dishes were incubated at 4°C for 30 min, rinsed in ice-cold phosphate-buffered saline (Dulbecco & Vogt, 1954) and re-incubated at 37°C for 30 min. After examination of the monolayers by phase-contrast microscopy, the buffered saline was replaced by hypo-osmotic buffer (5 mM-sodium phosphate buffer, pH 7.4) and the monolayers were re-examined by phase-contrast microscopy.

Polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970). Samples were either dialysed against a mixture containing 15% (v/v) glycerol in 1 M-Tris/HCl buffer, pH 6.8, or suspended in this mixture; sodium dodecyl sulphate (final concn. 2%, w/v), Bromophenol Blue (final concn. 0.2%) and dithiothreitol (final concn. 0.1 M) were added and the sample was heated in a boiling-water bath for 2 min. The stacking gel was 5% acrylamide and the running gel was 7.5% acrylamide.

Uptake of fluorescently labelled peptides was measured as follows. Leucine, trileucine (Leu-Leu-Leu), trityrosine (Tyr-Tyr-Tyr), pentaphenylalanine (Phe-Phe-Phe-Phe-Phe) (Sigma Chemical Co.) and vasopressin (CP Laboratories, Bishops Stortford, Herts., U.K.) were labelled with fluorescamine

(Udenfriend *et al.*, 1972) as follows. The amino acid or peptide (2 μ mol) was dissolved in 0.2 ml of phosphate-buffered saline (Dulbecco & Vogt, 1954) adjusted to pH 8 with NaOH, and 0.02 ml of fluorescamine (Sigma Chemical Co.) in ethanol (5 mg/ml) was added rapidly with shaking. After 15 min at room temperature, each sample (0.05 ml) was added to 0.5 ml of a suspension (1.2×10^7 cells/ml) of Lettrée cells that had previously been treated with or without virus (200 haemagglutination units/ml) for 10 min at 37°C. Incubation was continued at 37°C, and samples (0.1 ml) were removed at intervals and centrifuged through oil (Impraim *et al.*, 1979); each cell pellet was resuspended in 1 ml of phosphate-buffered saline, sonicated briefly and its fluorescence intensity at 465 nm (excitation wavelength 385 nm) measured with the instrument described above.

Results and discussion

Components required for permeability changes

In principle, two approaches are possible. The first is to disaggregate the virus into its constituent molecules and to test which of these is required to elicit permeability changes. The second is to degrade or inactivate specific components of intact virus and to determine whether such modifications inactivate the virus.

The first approach, though more straightforward, proved difficult to execute: a preparation showing permeability changes that can unequivocally be ascribed to viral components has not yet been obtained. The difficulties are threefold: first, to remove sufficient of the detergent that is required to solubilize the envelope proteins, yet retain sufficient phospholipids (Hosaka & Shimizu, 1972) to provide a milieu conducive to membrane fusion; secondly, to reconstitute the 'pores' (see below) present in intact virus; thirdly, to reassemble particles of a sufficient size. Although Hosaka & Shimizu (1972) and Volsky & Loyter (1978b) claim to have successfully reassembled preparations showing haemolysis or cell-cell fusion, and hence by implication (Knutton & Pasternak, 1979; Impraim *et al.*, 1980) permeability changes also, the retention of trace amounts of detergent was not rigorously excluded. Yet as little as 0.001% Triton X-100 is capable of causing permeability changes leading to haemolysis (Table 1). Moreover such changes are, like virally mediated permeability changes (Pasternak & Micklem, 1974a), inhibited by Ca^{2+} ; the same is true, at higher concentrations, of Nonidet (Table 1). Only by using the criterion of heat-sensitivity can any haemolysis genuinely be ascribed to the participation of viral components. Dialysis of Triton-extracted preparations against SM2 beads (Hollo-

way, 1973), for example, does not entirely remove all detergent (Table 1). Dialysis of Nonidet-extracted preparations is successful, however, though the resulting envelope particles, which contain only F and HN glycoproteins as shown by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, do not show permeability changes or haemolysis (Table 1). They do, however, show fluorescence enhancement (Fig. 3), which is likely to be a measure of vesicle fusion with Lettrée cells (see below). The envelope particles thus resemble '1-day' virus, which also shows fluorescence enhancement with Lettrée cells (Fig. 3) but does not cause permeability changes (Table 2) or haemolysis (Homma *et al.*, 1976); the envelope particles differ from '1-day' virus in that freeze-thawing does not promote permeability changes or haemolysis (cf. Table 1 with Table 2). Hsu *et al.* (1979) have obtained reassembled vesicles that are essentially free of detergent; in their case the vesicles, which are considerably larger, are able to induce haemolysis, and it will be interesting to compare the two preparations further.

The second approach for identifying the viral

Fig. 1. Effect of proteolytic treatment on viral components

Sendai virus (2 mg/ml) was exposed to trypsin or *S. aureus* proteinase for 1 h at 35°C, chilled, spun, resuspended in 5% (v/v) glycerol/0.1 M-Tris/HCl buffer, pH 6.8, containing 0.1 mM-phenylmethanesulphonyl fluoride, solubilized in sodium dodecyl sulphate and analysed by polyacrylamide-gel electrophoresis as described in the Methods and materials section. Key: track 1, untreated virus; track 2, trypsin-treated (20 μ g/ml); track 3, trypsin-treated (75 μ g/ml); track 4, *S. aureus*-proteinase-treated (10 μ g/ml); track 5, *S. aureus*-proteinase-treated (20 μ g/ml); track 6, untreated '1-day' virus. Key to bands: p, RNA polymerase; hn, haemagglutinin/neuraminidase; np, nucleoprotein; f, fusion factor; m, membrane protein.

Table 1. *Activity of reassembled envelope preparations*

Envelope preparations were made and assayed for phosphoryl[^3H]choline leakage, for haemagglutination, for haemolysis and for fluorescence enhancement as described in the Methods and materials section; for comparison, detergents were tested for leakage and haemolysis under the same conditions. N.D., Not determined.

Preparation	Leakage (% of cellular ^3H in medium)	Haemagglutination (haemagglutination units/ml)	Haemolysis (%)	Fluorescence enhancement (arbitrary units)
Virus	82	2000	100	80
Heated virus	26	<2	≤5	20
Reassembled envelope (prepared in Triton)	70	*	100	N.D.
Heated reassembled envelope (prepared in Triton)	69	*	100	N.D.
Reassembled envelope (prepared in Nonidet)	26	2000	≤5	80
Heated reassembled envelope (prepared in Nonidet)	25	<2	≤5	30
Reassembled envelope from trypsin-treated virus (prepared in Nonidet)	25	2000	≤5	30
0.0001% Triton	22	N.D.	≤5	N.D.
0.0001% Triton plus 10 mM- Ca^{2+}	20		N.D.	
0.001% Triton	52		≤5	
0.001% Triton plus 10 mM- Ca^{2+}	28		N.D.	
0.01% Triton	93		100	
0.01% Triton plus 10 mM- Ca^{2+}	90		N.D.	
0.001% Nonidet	22		≤5	
0.001% Nonidet plus 10 mM- Ca^{2+}	22		N.D.	
0.01% Nonidet	29		5	
0.01% Nonidet plus 10 mM- Ca^{2+}	25		N.D.	
0.02% Nonidet	53		5	
0.02% Nonidet plus 10 mM- Ca^{2+}	26		N.D.	
0.1% Nonidet	84		100	
0.1% Nonidet plus 10 mM- Ca^{2+}	79		N.D.	

* Could not be determined as residual detergent haemolysed the erythrocytes used in the assay.

components required for permeability changes has proved more fruitful. It is based on the findings that trypsin degrades F glycoprotein in preference to HN glycoprotein (Neurath *et al.*, 1973; Shimizu & Ishida, 1975), whereas a proteolytically active extract of *S. aureus* degrades HN glycoprotein in preference to F glycoprotein (M. Gething, personal communication). We have confirmed these findings (Fig. 1) and have tested the ability of enzymically modified Sendai virus to cause permeability changes, assessed by leakage of [^3H]choline-derived metabolites (Impraim *et al.*, 1980) from prelabelled Lettrée cells. Note that, at higher concentrations of trypsin or *S. aureus* extract, proteins other than F and HN glycoproteins (e.g. RNA polymerase) begin to be degraded.

The effect of exposing virus to trypsin is shown in Fig. 2(a); the effect of exposure of *S. aureus* proteinase is shown in Fig. 2(b). It is clear that trypsin, though having relatively little effect on the haemagglutination activity of the virus, markedly inhibits permeability changes. *S. aureus* proteinase, on the other hand, destroys haemagglutination activity under conditions where permeability changes are relatively unaffected. From this finding, taken together with the results of Fig. 1, it is clear that intactness of F glycoprotein is a more stringent requirement for permeability changes than is intactness of HN glycoprotein. Whether any HN glycoprotein is required at all (a > 80% decrease in haemagglutination activity was without effect in the present experiments; see Fig. 2b) could not be

Fig. 2. Effect of proteolytic treatment on biological activity of virus

Sendai virus (2 mg/ml) was exposed to trypsin or *S. aureus* proteinase for 1 h at 35°C at the final concentrations indicated, chilled, spun, resuspended in the same volume of Hepes-buffered saline and assayed for haemagglutination, haemolysis and phosphoryl³H]choline leakage as described in the Methods and materials section. (a) Trypsin treatment; (b) *S. aureus*-proteinase treatment. ●, Haemagglutination; ■, haemolysis; ▲, leakage.

determined, as prolonged incubation with *S. aureus* proteinase begins to degrade F glycoprotein also. Since the biological activity of F glycoprotein can be realized in the absence of HN glycoprotein, provided that some other agent is used to bring F glycoprotein into close contact with cells (e.g. Yamamoto & Inoue, 1978; Hsu *et al.*, 1979), it is likely that HN glycoprotein performs a purely permissive role during virally mediated permeability changes. Note that virus derived from cultured MDBK cells, which is non-infective because an inactive precursor of F glycoprotein, termed F₀ glycoprotein, is present instead of F glycoprotein (Scheid & Chopin, 1974; Ishida & Homma, 1978), does not elicit permeability changes; treatment of such virus with trypsin, which converts F₀ glycoprotein into F glycoprotein and renders it infective (Homma & Ohuchi, 1973), makes it active as regards permeability changes also.

Since we have suggested that the haemolytic action of Sendai virus is due to permeability changes (Knutton *et al.*, 1976; Impraim *et al.*, 1980), the effects of proteolytic treatment on permeability should be reflected by altered haemolysis. This is documented in Fig. 2, and confirms the initial observations with regard to proteolytic degradation (Neurath *et al.*, 1973; Shimizu & Ishida, 1975).

Permeability changes elicited by '1-day' virus

Virus harvested after growth in eggs for only 1 day is non-haemolytic and does not mediate giant-cell formation, but agglutinates cells and is infective (Homma *et al.*, 1976). The '1-day' virus does not elicit permeability changes, assessed either by leakage of phosphorylated metabolites from cells or by increased uptake of substances entering cells by simple diffusion (Table 2); the latter test includes the

uptake of bivalent cations such as Ca²⁺ and Mn²⁺ (Impraim *et al.*, 1979). Treatments that render such virus haemolytic, and capable of promoting giant-cell formation, such as freeze-thawing or incubation at 37°C (Shimizu *et al.*, 1976), cause it to elicit permeability changes also (Table 2; Impraim *et al.*, 1979). Note that uptake of α -methyl glucoside or of choline at high concentration is, like leakage of phosphoryl³H]choline, a measure of simple diffusion; uptake of 2-aminoisobutyrate or 2-deoxyglucose at low concentrations is, respectively, a measure of the integrity of the Na⁺ pump and of the ability to accumulate phosphorylated metabolites (Impraim *et al.*, 1980). Since '1-day' virus contains an F glycoprotein that is indistinguishable from that of '3-day' virus (Fig. 1), it is clear that the disposition of F glycoprotein within the viral envelope is an important factor in determining the ability to induce permeability changes. The '1-day' virus, unlike that modified by heating at 37°C, does not show the 14–15 nm intramembraneous particles (Kim *et al.*, 1979) typical of '3-day' virus (Knutton, 1976); '1-day' virus is not permeable to negative stains under conditions where the stain enters '3-day' virus (Shimizu *et al.*, 1976). Thus it is likely that some kind of 'pore' (Hosaka & Shimizu, 1977; Carrasco, 1977; Durham, 1977), perhaps represented by the 14–15 nm intramembraneous particles, is a requisite for eliciting permeability changes.

Size of 'pore' giving rise to increased permeability

The 'pores' through which an increased movement of ions, metabolites and phosphorylated derivatives takes place (Pasternak & Micklem, 1973, 1974a,b; Poste & Pasternak, 1978; Impraim *et al.*, 1979,

Table 2. *Comparison between '1-day' and '3-day' virus*

Leakage of phosphoryl[^3H]choline and uptake of labelled substrates (all at $1\mu\text{M}$ except [^3H]choline, which was at 20mM) were measured as described in the Methods and materials section. Virus for leakage experiments was 60 haemagglutination units/ml (final concentration), except for 37°C treatments, where it was 30 haemagglutination units/ml. Virus for uptake experiments was 100 haemagglutination units/ml; it was present for 30 min at 37°C before measurement of uptake at 37°C . The values quoted are expressed relative to control values obtained in the absence of virus. The control values were as follows: 18% of cellular phosphoryl[^3H]choline leaked out in 20 min; of the labelled substrate present in the medium, the following amounts were taken up within 4 min: 2.1% of [$\text{U-}^{14}\text{C}$] α -methyl glucoside; 0.5% of [^3H]choline; 17% of 2-amino[^{14}C]isobutyrate; 60% of 2-deoxy[^3H]glucose. N.D., Not determined.

Preparation	Treatment	Leakage of phosphoryl- [^3H]choline	Uptake of substrates			
			[^{14}C] α -Methyl glucoside	[^3H]Choline	2-Amino- [^{14}C]isobutyrate	2-Deoxy[^3H]- glucose
Control	—	100	100	100	100	100
'3-day' virus	—	484	195	220	7	34
'1-day' virus	—	138	110	100	63	103
'1-day' virus	Freeze-thawed $\times 3$	N.D.	171	140	17	96
'1-day' virus	Freeze-thawed $\times 6$	480	181	220	11	57
'1-day' virus	Freeze-thawed $\times 10$	N.D.	152	220	10	44
'1-day' virus	Incubated at 37°C for 20 h	198	N.D.	N.D.	N.D.	N.D.
'1-day' virus incubated at 37°C for 20 h	Freeze-thawed $\times 7$	418	N.D.	N.D.	N.D.	N.D.

1980) is such as to exclude proteins: thus [^{14}C]-valine-labelled cells do not leak trichloroacetic acid-insoluble ^{14}C -labelled material, nor do such cells lose any lactate dehydrogenase (Poste & Pasternak, 1978). Moreover $^{51}\text{CrO}_4^{2-}$ -labelled cells, which contain radioactivity predominantly in the form of bound $^{51}\text{Cr}^{3+}$ (Sanderson, 1976), do not leak ^{51}Cr out, and Trypan Blue (mol.wt. 961) does not leak in. (Pasternak & Micklem, 1973). In order to assess the size of 'pore' through which leakage does occur, we have measured the uptake of a series of fluorescently labelled peptides (Fig. 3). The initial uptake by control cells probably represents binding to the cell surface. Fig. 3 shows that the 'cut-off' point is at a mol.wt. of approx. 1000; of course molecular weight alone is not the only factor in determining effective molecular size or susceptibility to leakage. Simpson *et al.* (1977) have measured the penetration of fluorescently labelled peptides across communicating junctions, which are similar in several respects to virally mediated 'pores' (Micklem & Pasternak, 1977; Impraim *et al.*, 1980), and found the 'cut-off' point to occur at a mol.wt. of about 1200–1900; they conclude that the size of the 'pore' is approx. 1–1.4 nm in diameter. Hence the size of virally mediated 'pores' is likely to be in the region of 1 nm in diameter.

Fluorescence-enhancement method for assay of F glycoprotein

The best assay method for measuring fusion

between paramyxovirus and cells is probably that of Maeda *et al.* (1975, 1977), in which the behaviour of spin-labelled phospholipid, previously incorporated into cell membrane or into viral envelope, is measured by e.p.r. techniques; by using such methods it is possible to measure any phospholipid exchange that occurs at the same time as membrane fusion (Maeda *et al.*, 1977). Keller *et al.* (1977) have measured cell-cell fusion by recording energy transfer between two fluorescent probes, one of which is embedded in the plasma membrane of one population of cells and the other in another population of cells; this type of assay method (which does not distinguish between membrane fusion and lipid exchange) might be adapted for studying virus-cell fusion also. The fact that fusion and lipid exchange are probably catalysed by the same protein, namely F glycoprotein (Maeda *et al.*, 1977), means that, so far as biological activity of F glycoprotein is concerned, the method of assay is not crucial. Because e.p.r. measurements require rather costly apparatus as well as more material than fluorescence measurements, the latter have a certain advantage.

In exploring the mechanism by which fluorescent probes such as anthroylstearate are transferred from virus to cells during virus-cell fusion, we noticed that transfer is accompanied by a substantial increase in fluorescence intensity (Fig. 4). The increase is probably due to the fact that, in virus saturated with anthroylstearate, fluorescence inten-

Fig. 3. Uptake of fluorescently labelled amino acid derivatives

Uptake by cells previously treated with (■) or without (□) virus was measured as described in the Materials and methods section. (a) Fluorescamine-labelled leucine (mol.wt. 409); (b) fluorescamine-labelled trileucine (mol.wt. 636); (c) fluorescamine-labelled trityrosine (mol.wt. 786); (d) fluorescamine-labelled pentaphenylalanine (mol.wt. 1032); (e) fluorescamine-labelled vasopressin (mol.wt. 1278).

sity is quenched; as anthroylstearate is removed from the virus during its transfer to cells, quenching is diminished and fluorescence intensity is increased. At low concentrations of anthroylstearate in virus, at which there is no quenching, there is no enhancement during transfer to cells. If enhancement were to correlate with transfer of anthroylstearate to cells, and to occur only under conditions conducive to membrane fusion, it would make a useful yet simple assay method; Table 3 shows this to be so. Another advantage of this assay method is that, in anthroylstearate, the fluorescent grouping is well within the hydrophobic portion of the molecule, whereas in the probes used by Keller *et al.* (1977) the groupings are at the hydrophilic end of fatty acids. Because fluorescence intensity is dependent on several factors, such as temperature, the presence of extraneous proteins etc., it is best expressed relative to

some control value. We have used the enhancement that occurs in the presence of concanavalin A as 'base-line' control (Fig. 4). The fact that there is no enhancement in the presence of concanavalin A, which agglutinates cells, shows that virally mediated agglutination is not the cause of the enhancement.

The increase in fluorescence intensity is dependent on the possession of an intact F glycoprotein: virus containing trypsin-treated F glycoprotein does not show enhancement (Fig. 5). Treatment with *S. aureus* proteinase has relatively little effect. Enhancement is diminished at temperatures below 20°C, and is inhibited by heating and by addition of anti-(Sendai virus) serum, as well as by concanavalin A. Since the conditions under which the enhancement of fluorescence occur are also those under which transfer of anthroylstearate to cells takes place (Table 3), it is likely that some kind of

Fig. 4. Fluorescence enhancement by viral preparations

Various viral preparations (100 haemagglutination units) were treated with anthroylstearate as described in the Methods and materials section, and fluorescence intensity was measured. (a) ●, Virus; ■, heated virus; Δ, virus plus concanavalin A (150 μg/ml); ▲, virus plus antiserum; ○, '1-day' virus. (b) ●, Reassembled envelope preparations; ○, reassembled envelope preparations heated at 100°C for 2 min.

Table 3. Comparison between fluorescence enhancement and anthroylstearate transfer

Enhancement and transfer were measured, at 35°C unless stated otherwise, as described in the Methods and materials section. Proteolytic treatment was for 1 h with 75 μg of trypsin/ml or with 20 μg of *S. aureus* proteinase/ml. N.D., Not determined.

Condition	Enhancement (arbitrary units)	Transfer (arbitrary units)
Virus	100	100
Virus at 12°C	45	40
Virus at 15°C	60	60
Virus at 22°C	70	70
Heated virus	20	19
Virus and anti-(Sendai virus) serum	20	20
Virus and concanavalin A (150 μg/ml)	20	20
Virus and Ca ²⁺ (5 mM)	100	100
Virus and Ca ²⁺ (10 mM)	100	100
Trypsin-treated virus	25	20
<i>S. aureus</i> -proteinase-treated virus	80	N.D.

fusion event is occurring. One of the characteristics of membrane fusion is a temperature-dependence that is manifested by a hyperbolic curve when the reciprocal of 'time required for fusion to commence' is plotted against temperature (Ahkong *et al.*, 1973; see also Fig. 5 of Maeda *et al.*, 1975). Fig. 6 shows this to be true of the anthroylstearate enhancement assay also. A similar curve is obtained when 'time required for permeability change to commence' (Pasternak & Micklem, 1973) is measured; note that the presence of anthroylstearate in the viral envelope does not affect the 'time to permeability change' (Fig. 6). Fluorescence

enhancement appears to have a lower temperature threshold than leakage (Fig. 6).

If the enhancement assay is indeed a measure of intact F glycoprotein, it should occur with '1-day' virus as well as with '3-day' virus (see Fig. 1). Fig. 4 shows this to be so. Moreover reassembled envelope preparations, containing only F and HN glycoproteins and some lipid, are active in the fluorescence enhancement assay (Fig. 4b). Preparations containing only F or HN glycoproteins have not yet been tested: trypsin treatment abolishes fluorescence enhancement (Table 1), and this, coupled with the results obtained by Hsu *et al.* (1979), makes it

Fig. 5. Effect of proteolytic treatment on fluorescence enhancement

Sendai virus (2mg/ml) was exposed to trypsin (200µg/ml) for various periods of time or to *S. aureus* proteinase at the concentrations indicated for 1h at 35°C, chilled, spun, resuspended in HEPES buffered-saline and labelled with anthroylstearate as described in the Methods and materials section. Haemagglutination, and phosphoryl[³H]choline leakage and fluorescence enhancement at 15 min (at 25°C), were measured as described in the Methods and materials section. (a) Trypsin treatment; (b) *S. aureus*-proteinase treatment. ●, Haemagglutination; ○, fluorescence intensity; ■, leakage.

likely that F glycoprotein, rather than HN glycoprotein, is the active component.

The extent to which fluorescence enhancement is measuring transfer of anthroylstearate from virus to cells without membrane fusion has not been assessed. Although F glycoprotein has phospholipid-exchange activity (Maeda *et al.*, 1977), that activity is, by analogy with phospholipid-exchange protein (Wirtz, 1974), likely to be specific for phospholipids and not to be manifest with derivatives of fatty acids such as anthroylstearate or the probes used by Keller *et al.* (1977). One may

Fig. 6. Effect of temperature on fluorescence enhancement

The time taken for fluorescence enhancement or phosphoryl[³H]choline leakage (measured as described in the Methods and materials section) to become apparent was measured at different temperatures. ●, Fluorescence enhancement; ▲, leakage caused by anthroylstearate-labelled virus; ○, leakage caused by virus.

tentatively conclude that fluorescence enhancement is a measure of membrane fusion. If so, the characteristics of the assay [requirement for intact F glycoprotein, specific temperature-dependence, Ca²⁺-insensitivity (see Fig. 7)] should be shown by a direct optical assay of membrane fusion also. The following results confirm this prediction.

Optical method for assay of virus-cell fusion

Virus-cell fusion is difficult to assay by visual means. Although virally mediated cell-cell fusion has been assayed visually since its discovery by Okada (1958), such fusion is the consequence of several discrete stages that follow virus-cell fusion, and therefore not a true assay for virus-cell fusion itself (Knutton & Pasternak, 1979). Although electron microscopy is undoubtedly the most rigorous test of virus-cell fusion (e.g. Knutton, 1978), an optical method has distinct advantages. The discovery that fusion between '1-day' virus and erythrocytes leads to partially fused cells (cells joined by cytoplasmic bridges), and that from such

cells fully fused giant cells (i.e. polykaryons in the case of nucleated cells) can be formed merely by exposure to hypo-osmotic media (Knutton & Bachi, 1980), forms the basis of the present optical method, as shown in Plate 1.

With this method it can be seen that its characteristics are similar to those of the fluorescence-enhancement assay: activity with '1-day' virus, requirement for intact F glycoprotein (as shown by inhibition by trypsin treatment), diminished requirement for HN glycoprotein (as shown by relative insensitivity to treatment by *S. aureus* proteinase), insensitivity to 10 mM- Ca^{2+} and typical temperature-dependence (Table 4). In short, the assay exhibits the properties expected of a virus-cell fusion assay, in contrast with a giant-cell-formation assay. However, it has to be borne in mind that the assay method depends on effective cross-linking of cells by virus; at low concentrations of cells, or with reconstituted preparations that lack HN glycoprotein entirely or are too small to form effective cross-bridges, the assay method is ineffective.

Ca²⁺ effect on virus-cell fusion

Ca^{2+} inhibits permeability changes (Pasternak & Micklem, 1974a; Pasternak *et al.*, 1976; Micklem & Pasternak, 1977; Impraim *et al.*, 1979, 1980). In intact virus, these changes appear to be dependent on F glycoprotein and, to a smaller extent, on HN glycoprotein (Fig. 2). Does Ca^{2+} have any effect on the biological activity of F and HN glycoproteins? Table 5 and Fig. 7 show that Ca^{2+} , up to 10 mM, has

no effect on HN glycoprotein, as measured by agglutination of erythrocytes or of Lettrée cells; note that iodination of virus does not affect agglutination or haemolysis. This direct assay confirms previous assumptions with regard to insensitivity of agglutination to Ca^{2+} (Okada & Muryama, 1966); note that this does not appear to be true of all paramyxoviruses (Kobayashi, 1978). Likewise F glycoprotein, as measured by fluorescence enhancement (Fig. 7) or by giant-cell formation (Table 4), is not inhibited by Ca^{2+} at concentrations up to 10 mM, nor is it inhibited by EDTA.

These results, and those previously reported (Impraim *et al.*, 1979), therefore show quite clearly that exogenous Ca^{2+} is not required for membrane fusion between viral envelope and cell plasma membrane. On the basis of the observation that Ca^{2+} entry into erythrocytes is increased during chemically induced cell-cell fusion, Blow *et al.* (1979) have suggested that entry of Ca^{2+} is a necessary event for membrane fusion; others have made a similar suggestion with regard to virally induced (Volsky & Loyter, 1978b) and chemically induced (Lucy, 1978) cell-cell fusion. However, it is much more likely that Ca^{2+} entry is a consequence, not a cause, of an altered membrane, since chemical fusogens increase membrane permeability in general, as demonstrated by Knutton & Pasternak (1979) and by Blow *et al.* (1979) themselves. Moreover EDTA does not abolish cell-cell fusion of human erythrocytes (Quirk *et al.*, 1978). What is probably true is that the stability of giant cells (polykaryons) requires the presence of some Ca^{2+} ,

Table 4. *Effect of proteolytic treatment on virus-cell fusion*

Proteolytic treatment was carried out as described in the Methods and materials section. Haemagglutination, haemolysis and fusion were assayed as described in the Methods and materials section.

	Haemagglutination (haemagglutination units/ml)	Haemolysis (% of water lysis)	Virus-cell fusion (% of cells undergoing fusion)
Virus	1000	100	40
Trypsin-treated virus (200 µg/ml)			
(5 min)	1000	55	<5
(10 min)	1000	28	1-2
(20 min)	1000	5	0
(50 min)	160	0.3	0
Virus	1280	100	40
<i>S. aureus</i> -proteinase-treated virus			
(5 µg/ml)	640	100	40
(10 µg/ml)	640	100	30-40
(20 µg/ml)	320	98	20
(30 µg/ml)	160	56	<5
(35 µg/ml)	80	45	<1
'1-day' virus	1000	8	40
'1-day' virus tested at 20°C	1000	0	2
'1-day' virus plus 10 mM- Ca^{2+}	1000	0	40
'1-day' virus plus 2 mM-EDTA	1000	7	40

EXPLANATION OF PLATE 1

Optical assay of virus-cell fusion

Phase-contrast micrographs showing monolayers of human erythrocytes treated with '1-day' Sendai virus at 37°C for 30 min (a) and the same cells subsequently exposed to hypo-osmotic medium (b). Membrane fusion between many cells has occurred in (a) but is only apparent after an experimental hypo-osmotic swelling (b). Magnification $\times 500$.

Table 5. *Effect of Ca²⁺ on binding of ¹²⁵I-labelled virus to cells*

Lettrée cells (0.2 ml at 4×10^7 /ml) were incubated for 15 min at 4°C or 35°C with 0.05 ml of ¹²⁵I-labelled virus (100 haemagglutination units) under the conditions stated, and haemagglutination, binding and haemolysis were assayed as described in the Methods and materials section.

Preparation	Addition (final concn.)	Temperature (°C)	Haemagglutination (haemagglutination units/0.05 ml)	Binding (% of that bound at 4°C)	Haemolysis (% of that at 35°C)
¹²⁵ I-labelled virus	—	4	100	100	≤5
¹²⁵ I-labelled virus	5 mM-Ca ²⁺	4	100	73	≤5
¹²⁵ I-labelled virus	10 mM-Ca ²⁺	4	100	87	≤5
¹²⁵ I-labelled virus	—	35	100	34	100
¹²⁵ I-labelled virus	5 mM-Ca ²⁺	35	100	41	40
¹²⁵ I-labelled virus	6 mM-Ca ²⁺	35	100	38	40
¹²⁵ I-labelled virus	10 mM-Ca ²⁺	35	100	41	10
¹²⁵ I-labelled virus	Antiserum (diluted 12-fold)	35	<0.1	12	≤5
¹²⁵ I-labelled virus	Concanavalin A (150 µg/ml)	35	100	>100	≤5
Trypsin-treated ¹²⁵ I-labelled virus	—	35	100	34	≤5
Heated ¹²⁵ I-labelled virus	—	35	<0.1	10	≤5
Virus	—	4	100		≤5
Virus	—	35	100		100
Virus	5 mM-Ca ²⁺	35	100		40
Virus	10 mM-Ca ²⁺	35	100		≤5

Fig. 7. *Effect of Ca²⁺ on fluorescence enhancement*

Fluorescence enhancement and phosphoryl[³H]choline leakage, at the final concentrations of CaCl₂ indicated, were measured as described in the Methods and materials section. ●, Fluorescence enhancement; ▲, leakage; ○, haemagglutination.

since EDTA appears to be inhibitory to the formation of polykaryons (Pasternak & Micklem, 1974a).

General discussion and conclusions

The results given in the present paper are in accord with the hypothesis previously advanced (Knutton & Pasternak, 1979). First, that perme-

ability changes result from virus-cell fusion, which is itself dependent on the presence of intact F glycoprotein; the demonstration that a specific viral component (F glycoprotein) is required for permeability changes rules out the possibility that the changes are due to contaminants in the viral preparation, to interferon or to other products. Secondly, the results show that Ca²⁺-sensitivity of haemolysis and giant-cell formation (Impraim *et al.*,

Scheme 1. Stages in virally mediated membrane fusion

1980) is due to inhibition of permeability changes, and not to inhibition of virus-cell binding (Table 5) or virus-cell fusion (Table 4 and Fig. 7).

Virus-cell fusion is clearly not the only prerequisite for permeability changes, since '1-day' virus and reconstituted viral envelope undergo fusion with cells but do not cause permeability changes. The results with modified '1-day' virus (Table 2) make it likely that some kind of leaky envelope is a requirement. Leakiness might be the result of a cluster of viral glycoproteins that, when fused with cells, form a hydrophilic channel somewhat analogous to that formed by polypeptide ionophores (Carrasco, 1977; Durham, 1977). That is, the sequence of events is as depicted in Scheme 1. Stage 1 is assayed by binding of ^{125}I -labelled virus to cells or by haemagglutination; stage 2 is assayed by fluorescence enhancement or by the optical virus-cell-fusion assay method described in the present paper (or by electron microscopy; Knutton, 1978); stage 3 is assayed by leakage, by haemolysis or by giant-cell formation.

The mechanism by which HN glycoprotein brings cells and virus together is by binding to neuraminic acid-containing residues on the cell surface (e.g. Bachi *et al.*, 1977). The mechanism by which F glycoprotein causes fusion is less clear. The demonstration that F glycoprotein catalyses phospholipid exchange as well as causing membrane fusion (Maeda *et al.*, 1977) may be relevant. For phospholipid transfer may be the basis by which instability in apposed membranes, leading to fusion, is achieved (Papahadjopoulos, 1978). In that case, the biological activity of F glycoprotein may be that of a phospholipid-exchange protein (Maeda *et al.*, 1977). Since it has been suggested that the physiological role of phospholipid-exchange proteins found in the cytoplasm of cells is also to cause membrane fusion (Quinn & Pasternak, 1980), a unified scheme for the relation between phospholipid exchange and membrane fusion follows. Phospholipid-exchange proteins in the cytoplasm of cells cause fusion between intracellular membranes, such as between Golgi vesicles and plasma membrane (exocytosis and membrane assembly), or between plasma membrane and lysosomes (endocytosis and membrane turnover); phospholipid-exchange proteins in the membrane of viruses (and perhaps in lipoprotein vesicles also) cause fusion between external vesicles and the plasma membrane.

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