J. Membrane Biol. 14, 293–303 (1973)

Permeability Changes during Cell Fusion

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Received 6 June 1973

Summary. During Sendai virus-mediated fusion of mouse ascites cells, there is a loss of intracellular metabolites; at the same time accumulation from the medium is inhibited. This failure to maintain selective permeability does not occur at 0 °C; it is unaffected by cytochalasin B, which inhibits fusion. It therefore represents a discrete, temperature-dependent event and may result from a weakening in membrane architecture necessary to achieve fusion.

Although the fusion of membranes is a key event in many biological phenomena such as pinocytosis, phagocytosis, secretion, cytokinesis, myogenesis or the entry and exit of membrane-coated animal viruses, its mechanism is unknown (Poste & Allison, 1971; Poste, 1972; Pasternak & Quinn, 1974). From studies of virus-mediated cell fusion it is clear that close proximity between membranes, achieved in this case by agglutination, is the first requirement. It has been suggested that some kind of membrane destabilization then triggers fusion. We have now obtained evidence that during cell fusion the plasma membrane loses its ability to maintain concentration differences of water-soluble metabolites and that this is a discrete event in the fusion process.

Experimental

Lettrée ascites cells were passaged in Swiss white mice and washed in tris-buffered (50 mM, pH 7.2) or phosphate-buffered saline (Dulbecco & Vogt, 1954) before use. Intracellular pools were labeled by intraperitoneal injection of isotope into ascites-bearing animals some hours earlier, or by incubation of cells *in vitro* in the presence of isotope ([methyl-^{3}H] choline chloride, 16.5 C/mmol; [methyl-^{14}C] choline chloride, 60 C/mol; [1-^{3}H] 2-deoxy D-glucose, 22 C/mmol).

Cells were incubated in tris- or phosphate-buffered saline with UV-irradiated (1,000 ergs/cm^2 per sec for 9 min) Sendai virus [900 to 2,700 haemagglutinin units (HAU)/ml] and fusion assessed microscopically; addition of Ca^{++} (1 mM) was without effect on fusion. Heat-killed (1 hr at 60 °C) virus was used as a control. The difficulty of

sampling clumped cells was avoided by distributing cell suspension into 0.1-ml portions in centrifuge tubes prior to addition of virus (0.025 to 0.05 ml). The tubes were incubated at various temperatures and the contents assayed by centrifuging rapidly and removing supernatant medium with a syringe. The cell pellet was either suspended in scintillation fluid (5 g PPO, 20 g naphthalene in 1 liter dioxan) and assayed directly, or extracted (a) in 1 ml of 5% ice-cold trichloroacetic acid and spun to yield a soluble and insoluble fraction or (b) in 2 ml of chloroform/methanol (2:1), spun, 0.4 ml water added to the supernatant, and the upper and lower layers separated. Radioactivity in trichloroacetic acid-soluble and -insoluble fractions (a), or in chloroform/methanol pellets, upper and lower layer (b), was determined. The variation in recovery of radioactivity from supernatant media or from fractioned cell pellets of four identical samples was less than 10%.

All experiments involving cytochalasin B (ICI Research Laboratories, Alderley Park, Cheshire) were carried out in the presence of 0.4% dimethylsulfoxide as solubilizing agent; this concentration was present in all controls and was without effect on any of the parameters under study.

Results

[^{3}H] choline-labeled cells show a virus-dependent loss of isotope from the trichloroacetic acid-soluble fraction into the medium (Fig. 1); like fusion itself, leakage of isotope begins more rapidly, and is faster, at 37 °C than at 22 °C. No isotope is lost at 0 °C, though agglutination is instantaneous. A more detailed analysis of the effect of temperature on the length of the lag period before leakage occurs reveals an inflection between 20 and 30 °C (Fig. 2*a*). Similar temperature-dependent changes have been observed by measuring the 'time to fusion' during the chemically-induced fusion of hen erythrocytes (Ahkong, Cramp, Fisher, Howell, Tampion, Verrinder & Lucy, 1973) or the mixing of surface antigens during the formation of man-mouse heterokaryons (Frye & Edidin, 1970). Since the overall phase transition of animal membranes appears to be nearer 0 °C (Blazyk & Steim, 1972) than 20 °C, these changes in membrane fluidity must be due to localized changes or else to causes other than lipid 'melting'. An Arrhenius plot of the rate of leakage of intracellular choline certainly reveals no discontinuities above 17 °C (Fig. 2*b*).

Leakage occurs irrespective of whether cells are labeled *in vivo* (e.g., Fig. 1) or *in vitro* (e.g., Fig. 3), between 10 and 50% of water-soluble isotope being released. This probably reflects the number of cells which fuse, rather than a limitation on the amount released per cell, though the possible existence of more than one intracellular pool of any one phospholipid precursor derived from choline has to be borne in mind. Paper chromatography (ethanol/1 M ammonium acetate, pH 5, 7:3) of chloroform/methanol upper layer of cells obtained from a mouse injected 16 hr previously with 10 μC [^{14}C] choline showed that >95% of the radioactivity is in a compound having the mobility of phosphorylcholine (*cf.* Plagemann, 1971;

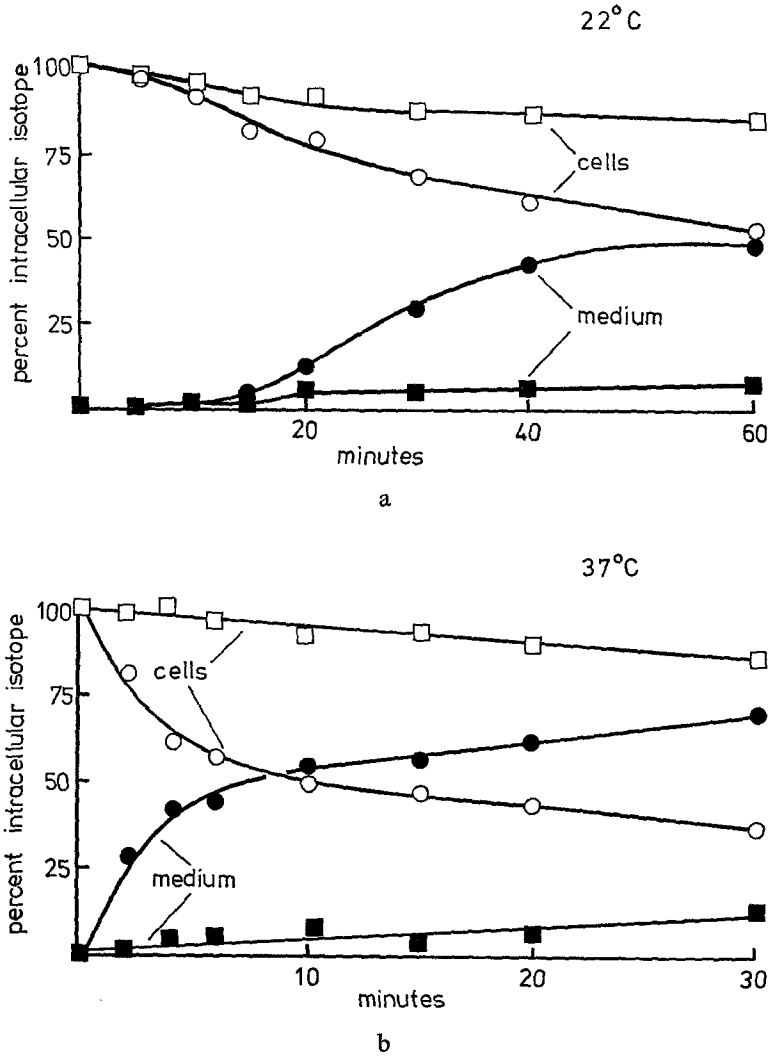

Fig. 1. Loss of intracellular [^{3}H] choline during fusion. Cells from a mouse injected 113 hr previously with 100 μC [^{3}H] choline were incubated (10^7 cells/ml) with virus (2,700 HAU/ml) and assayed as described in the Experimental section. Cell pellets were extracted with 5% trichloroacetic acid. Radioactivity in the trichloroacetic acid-insoluble fraction (approximately 60% of the total in the cells) did not change significantly during incubation. Closed symbols are [^{3}H] in medium; open symbols [^{3}H] in the trichloroacetic acid-soluble fraction, each expressed as a percentage of the total intracellular isotope. Normal virus •—• ○—○; heat-killed virus, ▪—▪ ▫—▫. (*a*) 22 °C; (*b*) 37 °C

Knox & Pasternak, 1973; Pasternak & Knox, 1973), and this is also the form (>97% of total released) in which isotope appears in the medium during fusion. Phospholipid turnover is not affected significantly during

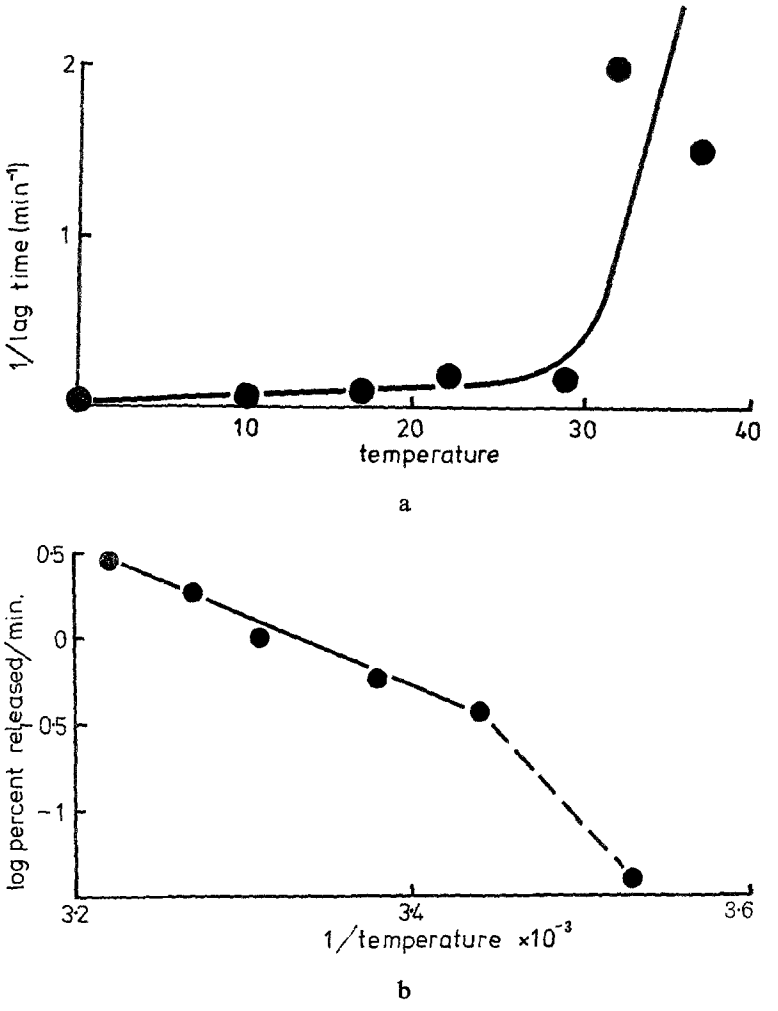

Fig. 2. Temperature-dependence of loss of intracellular [^{14}C] choline during fusion. Cells (10^8/ml) were incubated for 30 min at 37 °C with [^{14}C] choline (2.5 μC/ml; 10 μM), washed, resuspended (1.2×10^7/ml) and incubated with virus (1,600 HAU/ml) at various temperatures. Radioactivity in the medium and in the cell pellet without further extraction was measured as described in the Experimental section. The reciprocal of the time elapsing before appreciable leakage is discernible is plotted against temperature in (*a*). In (*b*), the log of the rate of leakage is plotted against the reciprocal of the absolute temperature (Arrhenius plot)

fusion; <1% of cellular lecithin, for example, is degraded to lysolecithin (Lucy, 1970) (<2 nmol/10^7 cells) or other products *(unpublished experiments)*. The number of phospholipid molecules involved in fusion, however, might be rather small (*cf.* Lachmann, Bowyer, Nicol, Dawson & Munn,

1973), and one cannot discount the participation of some kind of phospholipid or other turnover entirely (Pasternak & Quinn, 1974); on the one hand lysolecithin is formed during fusion at high pH (Toister & Loyter, 1973), while on the other lysolecithin actually inhibits fusion of myoblasts (Reporter & Norris, 1973).

Cells labeled with [^{3}H] inositol, [^{3}H] deoxyglucose or [^{32}P] inorganic phosphate show a similar release of water-soluble metabolites; leakage of inorganic ions is at present being investigated. Other intracellular components either do not leak out *(see below)*, or do so as fast with heat-killed as with intact virus (e.g., amino acids or glucosamine). In other words, compounds such as choline or deoxyglucose (Renner, Plagemann & Bernlohr, 1972) that are normally prevented from leaking out of cells by phosphorylation show the biggest effect.

Loss of intracellular components is not due to lysis, since [^{51}Cr] chromate-labeled cells do not release isotope (<3% lost during 30 min in presence of virus; 70% lost by water lysis) and fluorescein diacetate-labeled cells (Rotman & Papermaster, 1966) do not release fluorescein. Nor do fusing cells fail to exclude trypan blue (>93% of cells excluding trypan than after 20-min incubation in presence or absence of virus). This confirms electron-microscopic evidence showing the formation of cytoplasmic bridges rather than holes during fusion (Harris, 1970).

It might be argued that the loss of intracellular isotope is an artifact due to an increased fragility of plasma membrane during fusion, the isotope being lost only when cells are centrifuged during the assay procedure. This is unlikely, since cells placed on a membrane filter and allowed to drain without suction show the same effect (Table 1). Moreover, cell pellets resuspended after centrifugation still exclude trypan blue.

Cytochalasin B, which blocks other biological processes involving fusion (Davis, Estensen & Quie, 1971; Estensen, 1971; Taylor, Duffus, Raff & de Petris, 1971; Wagner, Rosenberg & Estensen, 1971; Wessels, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn & Yamada, 1971; Williams & Wolff, 1971; Orr, Hall & Allison, 1972), inhibits Sendai-mediated fusion of ascites cells at concentrations at which synthesis of phospholipid, protein or DNA is not appreciably affected (Table 2). Other studies have likewise shown that synthesis of phospholipid (Estensen, 1971), protein and RNA (Maslow & Mayhew, 1972) is relatively unaffected by cytochalasin B. Reports of decreased DNA (Estensen, 1971; Yoshinaga, Waksman & Malawista, 1972) and mucopolysaccharide (Sanger & Holtzer, 1972) synthesis are probably due to inhibition of transport rather than of incorporation (Estensen & Plagemann, 1972; Kletzien & Perdue, 1973). Like agglutina-

Table 1. Loss of intracellular [^{3}H] deoxyglucose and [^{14}C] choline during fusion

Treatment	Assayed by centrifugation (a)		Assayed by filtration (b)	
	Appearing in medium	Lost from cells	Appearing in medium	Lost from cells
	[^{3}H] (cpm)		[^{3}H] (cpm)	
Virus	15,000	8,000	15,000	2,000
Heat-killed virus	2,000	3,000	5,000	500
	[^{14}C] (cpm)		[^{14}C] (cpm)	
Virus	1,600	1,100	1,100	400
Heat-killed virus	500	300	100	30

The cells labeled with [^{3}H] deoxyglucose and [^{14}C] choline for the experiment described in Fig. 3 were used; total intracellular isotope at start was 20,000 cpm [^{3}H] and 1,900 cpm [^{14}C]. Duplicate samples of cells without cytochalasin B were incubated (1.2×10^7/ml) with virus (1,600 HAU/ml) for 0 and 21 min at 24 °C. One set of samples (a) was assayed by centrifugation as described in the Experimental section; radioactivity in the cell pellet without further extraction was measured. The other set of samples (b) was diluted with 10 ml of tris-buffered saline and allowed to leak through previously-wetted oxoid membrane filters without suction. Radioactivity on the filters and in the filtrate was measured; the low recovery of ^{3}H and ^{14}C lost from cells is due to counting problems.

Table 2. Effect of cytochalasin B on fusion (a) and precursor incorporation (b)

(a) Cytochalasin B (μg/ml)	Virus	Fusion index	Agglutination
0	heat-killed	<0.01	–
0	normal	0.28	+++
2	normal	0.03	+++
8	normal	0.02	+++
32	normal	<0.01	+++

(b) Cytochalasin B (μg/ml)	% of intracellular precursor incorporated			
	[^{14}C] choline	[^{3}H] inositol	[^{14}C] valine	[^{3}H] thymidine
0	15	54	79	92
40	22	53	69	85

(a) Cytochalasin B was added to cells (2×10^7/ml), followed by virus (1,600 HAU/ml) and incubated for 40 min at 24 °C. Samples were stained (Welshons, Gibson & Scandlyn, 1962) and the fusion index (number of fused cells/total cells) determined by scoring nuclei (>100 per field). Agglutination of +++ denotes >99% of cells clumped; – denotes <1% clumped.

(b) Incorporation of precursors was assessed by incubating cells (5×10^6/ml) for 40 min at 37 °C in the presence of cytochalasin B and either [methyl-^{14}C] choline chloride (16.5 C/mmol; 0.2 μC/ml) and [2-^{3}H] myo-inositol (2 C/mmol; 2 μC/ml) or [U-^{14}C] L-valine (280 C/mol; 0.2 μC/ml) and [methyl-^{3}H] thymidine (2 C/mmol; 2 μC/ml). Cells were spun and the pellets extracted with chloroform/methanol as described in the Experimental section. The radioactivity in the chloroform/methanol pellet and lower layer is expressed as a per cent of the total present in the cell.

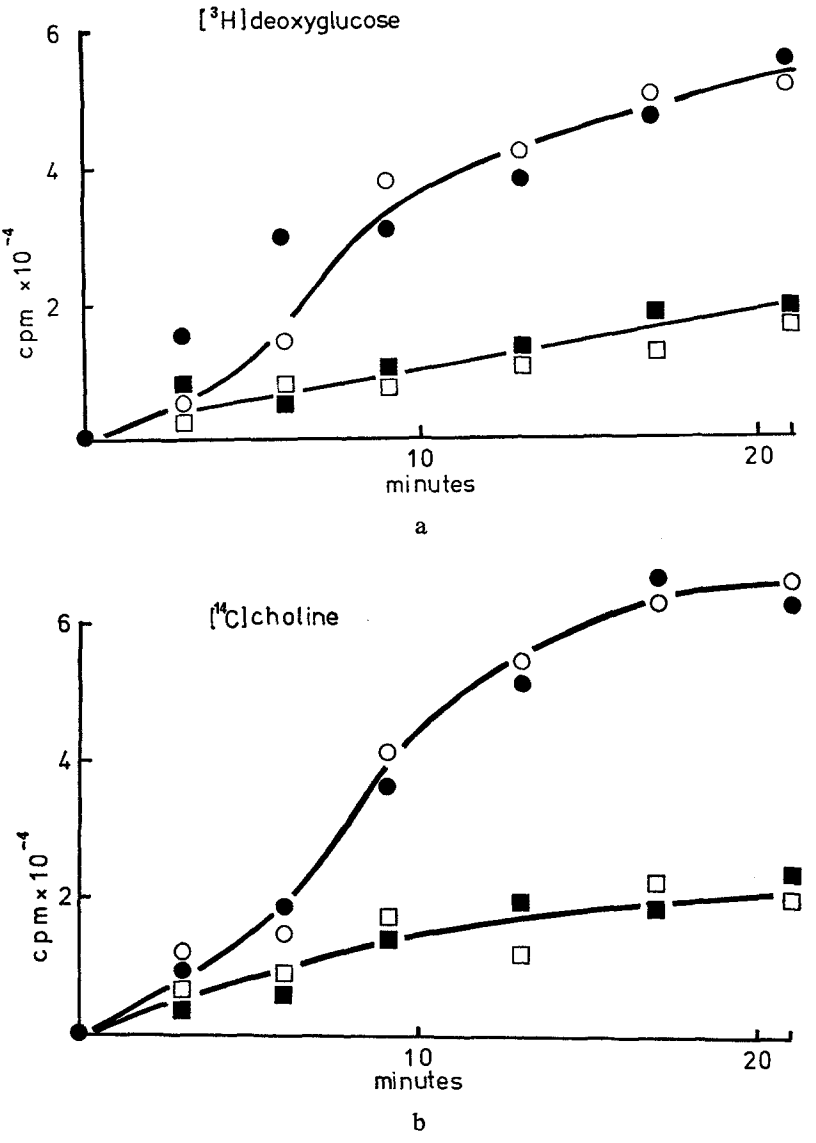

Fig. 3. Effect of cytochalasin B on loss of intracellular [^{3}H] deoxyglucose and [^{14}C] choline during fusion. Cells (5×10^7/ml) were incubated for 30 min at 35 °C with [^{3}H] deoxyglucose (20 µC/ml; 20 µM) and [^{14}C] choline (2 µC/ml; 8 µM), washed, resuspended (1.2×10^7/ml) with or without cytochalasin B (30 µg/ml) and incubated with virus (1,600 HAU/ml) at 24 °C. Radioactivity in the medium was measured as described in the Experimental section. Normal virus, •—• ○—○; heat-killed virus, ▪—▪ ▫—▫. Open symbols, without cytochalasin; closed symbols, with cytochalasin

tion (Table 2), leakage of intracellular components is insensitive to the presence of cytochalasin B (Fig. 3). If anything, leakage occurs a little more

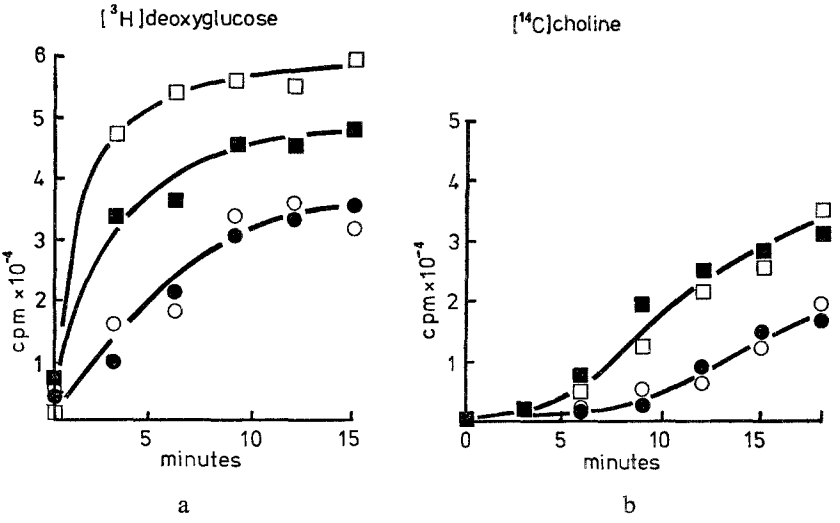

Fig. 4. Effect of cytochalasin B on uptake of [^{3}H] deoxyglucose and [^{14}C] choline during fusion. Cells suspended (3×10^7/ml) in the presence of [^{3}H] deoxyglucose (25 μC/ml; 20 μM) and [^{14}C] choline (2.5 μC/ml; 10 μM) with or without cytochalasin B (30 μg/ml) were incubated with virus (1,600 HAU/ml) at 37 °C. Samples were diluted with 2 ml of tris-buffered saline, allowed to drain through moistened GF/C filters with gentle suction, washed with 20 ml of tris-buffered saline and radioactivity on the dried filters assayed. Normal virus, •—• ○—○; heat-killed virus, ▪—▪ ▫—▫. Open symbols, without cytochalasin; closed symbols, with cytochalasin

rapidly, which may be of significance in relation to a report (Beck, Jay & Saari, 1972) of increased fragility of human erythrocytes in the presence of cytochalasin B. The important point about the present experiments, however, is that in the absence of intact virus, leakage is unaffected by cytochalasin B (Fig. 3). Although ascites cells show the morphological effects typical of the action of cytochalasin B (Wagner *et al.*, 1971), these changes clearly do not prevent the loss of intracellular components. Agglutinated cells in the presence of cytochalasin B may therefore be at a sort of 'halfway' stage (Poste & Allison, 1971) towards fusion.

If virus disrupts the process by which certain metabolites are retained within cells, it is also likely to affect the uptake of compounds from the medium. Fig. 4 shows that this is indeed the case. Note that, in confirmation of previous studies, uptake of deoxyglucose (Estensen & Plagemann, 1972; Kletzien, Perdue & Springer, 1972; Zigmond & Hirsch, 1972), but not of choline (Estensen & Plagemann, 1972), is inhibited by cytochalasin B. Superimposed on this inhibition, however, is the additional inhibition due to virus (Fig. 4). Uptake of [^{14}C]-2-amino-iso-butyric acid is likewise inhibited

by virus; as with choline, uptake is unaffected by cytochalasin B. Such experiments *per se* do not distinguish between the inhibition of an active uptake process and the stimulation of a passive exit as possible mechanisms of viral action. But the fact that the entry of phosphorylcholine is very slow, and insensitive to the presence of virus *(unpublished experiments)*, makes it likely that some kind of passive leakage is indeed involved.

Conclusion

It has been demonstrated that Sendai virus causes a permeability change in the plasma membrane which allows molecules below a certain size (e.g., phosphorylated derivatives of choline and deoxyglucose, but not chromium or fluorescein) to leak out. In that respect, virally-mediated fusion is different from the fusion occurring during myogenesis, in which selective permeability appears to be maintained (Rash & Fambrough, 1973). The release of intracellular macromolecules during viral infection (Gilbert, 1963; Cassells, 1973) occurs much later and is a different phenomenon. Leakage of intracellular components is not due to agglutination since it does not occur at 0 °C; on the other hand, the temperature-dependence of the effect (Fig. 2) shows a close similarity to that of fusion itself (Ahkong *et al.*, 1973 and present experiments). Leakiness is an event distinct from fusion however, since it is unaffected by cytochalasin B. It is therefore likely that it results from a virally-induced weakness of membrane structure, which may itself be a trigger for subsequent fusion.

Since loss of intracellular components is an easily-measured parameter of viral function, it should prove useful in fractionation studies of viral components, as well as in elucidating the molecular events by which membrane stability is disrupted so as to promote fusion. Studies with fluorescent and other membrane probes are currently in progress to investigate this point.

We are grateful to Professor H. Harris for supplying Sendai virus and for helpful discussion and to the Medical Research Council for financial support.

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