Nature of Virally Mediated Changes in Membrane Permeability to Small Molecules

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(Received 23 August 1979)

1. The changes in membrane permeability to small molecules caused by Sendai virus [Pasternak & Micklem (1973) *J. Membr. Biol.* **14**, 293–303] have been further characterized. The uptake of substances that are concentrated within cells is inhibited. Choline and 2-deoxyglucose, which become phosphorylated, and aminoisobutyrate and glycine, which are driven by a Na^+ -linked mechanism, are examples. The uptake of each compound under conditions where its diffusion across the plasma membrane is rate-limiting is stimulated by virus. Choline, 2-deoxyglucose and amino acids at high concentration, amino acids in Na^+ -free medium, and most substances at low temperature, are examples. It is concluded that virally mediated decrease of uptake is due to one of two causes. Substances that are accumulated by phosphorylation are not retained because of leakage of the phosphorylated metabolites out of cells. Substances that are accumulated by linkage to a Na^+ gradient are no longer accumulated because of collapse of the gradient resulting from an increased permeability to Na^+ . 2. Increased permeability to K^+ and Na^+ results in (a) membrane depolarization and (b) cell swelling. The latter event leads to haemolysis (for erythrocytes) and can lead to giant-cell (polykaryon) formation (for several cell types). 3. Recovery of cells can be temporarily achieved by the addition of Ca^{2+} ; permanent recovery requires incubation for some hours at 37°C . 4. The possible significance of virally mediated permeability changes, with regard to clinical situations and to cell biology, is discussed.

The initial event in viral attack, as well as the final stage in viral release, is an interaction with the cell surface. When paramyxoviruses such as Newcastle Disease virus (Klemperer, 1960b) or Sendai virus (Pasternak & Micklem, 1973) are added to cells, leakage of intracellular constituents takes place. Leakage is not due to lysis, and is restricted to the passage of low-molecular-weight compounds and ions, i.e. to an increase in simple diffusion (Pasternak & Micklem, 1973, 1974a). At the same time there is a decrease in the uptake of substances that become accumulated in cells following a facilitated transport mechanism. Virus thus appears to have an inverse effect on passive and facilitated uptake (Pasternak & Micklem, 1974b). Initial attempts to separate the two effects proved unsuccessful (Pasternak *et al.*, 1976), and it may be supposed that they are somehow linked. Here we show that a failure to retain accumulated compounds that enter by facilitated transport is not due to an effect on the uptake mechanism itself, but is a consequence of an increase in the permeability of cells.

An increase in simple diffusion of ions such as K^+ (Klemperer, 1960b; Fuchs & Giberman, 1973; Pasternak & Micklem 1974a) and Na^+ (Poste & Pasternak, 1978) is likely to result in membrane depolarization (Okada *et al.*, 1975). The nature of this change is documented below. Another outcome is an entry of water, resulting in a swelling of cells (Knutton *et al.*, 1976); we show here that this underlies not only the haemolytic action of paramyxoviruses, but also their capacity to mediate giant-cell (polykaryon) formation (Okada, 1958; Harris, 1970).

The effects of paramyxoviruses are prevented (Pasternak & Micklem, 1974a), and can be reversed (Pasternak *et al.*, 1976), by Ca^{2+} . Some aspects of the nature of the recovery process are documented in the present paper.

Materials and Methods

Sendai virus was grown in 10-day-old embryo-nated eggs and harvested after 3 days ('3-day' virus)

or after 1 day ('1-day' virus; Homma *et al.*, 1976); unless otherwise stated, all virus was of the '3-day' type. Occasionally virus grown for 3 days was found to have '1-day-like' character with respect to permeability changes and haemolysis; freeze-thawing, and dilution, of virus restored permeability changes and haemolysis. It is concluded that variables other than time of growth in eggs affects the capacity of Sendai virus to cause permeability changes. Haemagglutination units (HAU) of virus were determined by the method of Martin (1978).

Lettree cells were grown by serial passage in the peritoneal cavity of MF1 mice and harvested between 7 and 10 days. Cells were suspended in a solution containing 150 mM-NaCl, 5 mM-KCl and 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer adjusted to pH 7.2 with NaOH ('Na⁺ medium') unless stated otherwise. 'K⁺ medium' contained 155 mM-KCl and 5 mM-Hepes buffer adjusted to pH 7.2.

Radioactive compounds used were [U-¹⁴C]-adenosine (CFB 54), 2-amino[1-¹⁴C]isobutyric acid (CFA 203), [*Me*-¹⁴C]choline (CFA 424), 2-deoxy-D-[1-³H]glucose (TRK 383), 3-*O*-methyl-D-[U-¹⁴C]-glucose (CFB 141), [U-¹⁴C]glycine (CFB 66), [³H]inulin (TRA 324), L-[4,5-³H]leucine (TRK 170), methyl α -D-[U-¹⁴C]glucopyranoside (CFB 76), L-[3,4(n)-³H]valine (TRK 533) and L-[U-¹⁴C]valine (CFB 75), all from The Radiochemical Centre, Amersham, Bucks., U.K., and [*Me*-³H]choline (NET 109) from New England Nuclear, Boston, MA, U.S.A. The concentrations to which cell suspensions were exposed were 1–2 μ Ci of ³H/ml and 0.1–0.2 μ Ci of ¹⁴C/ml.

Permeability changes were assessed as follows. Leakage from pre-labelled cells was measured by the method of Pasternak & Micklem (1973, 1974a). Uptake of labelled substrates was measured either by washing cells in ice-cold buffer or by spinning through oil (Impraim *et al.*, 1979). The sample volume was 0.2 ml in each case.

Phosphorylated metabolites of [³H]choline, 2-deoxy[³H]glucose, [¹⁴C]adenosine and ³²P_i were measured as follows. Medium was separated from cells by spinning through oil, and cell pellets were suspended in water, sonicated at 20 kHz and 15 W with a 3 mm probe for 30 s at 5°C, and spun (2700 g-min) to obtain a water extract and residue. Alternatively cell pellets were extracted in 3%, (w/v) HClO₄ at –5°C, followed by centrifugation (2700 g-min) and removal of HClO₄ from the supernatant by precipitation with KOH. Medium and water extract (or HClO₄ extract) were analysed by paper chromatography [1 M-ammonium acetate (pH 5)/ethanol, 3:7, v/v] or by ion-exchange chromatography on Polygram CEL 300 PEI (polyethyleneimine) sheets (Macherey Nagel and Co., 516 Duren, Germany) paper, with 3 M-LiCl/1 M-acetic acid (1:9, v/v) as

solvent. Labelled material in the residue in [¹⁴C]-adenosine- and ³²P_i-labelled cells was largely labelled RNA and DNA.

Membrane potential was assessed by the use of a fluorescent cationic dye (3,3'-dipropylloxadicarbocyanine), kindly given by Professor A. A. Eddy, as described by Philo & Eddy (1978). The fluorescence intensity was measured with a Perkin Elmer MPF 44A spectrofluorimeter operated in the a.c. ratio mode to correct for lamp-output variations. Samples were excited at 580 nm, bandwidth 5 nm, and the emission was measured at 620 nm, bandwidth 5 nm, at 90° to the excitation beam.

Fig. 1. Effect of virus on uptake of various substances. Lettree cells were incubated at 37°C with or without virus in the presence of radioactive substrates at the concentrations indicated, and uptake was assessed as described in the Materials and Methods section. Data from a number of different experiments are combined. (a) ●, Choline; ○, glycine; ■, aminoisobutyrate; □, 2-deoxyglucose; (b) ●, α -methyl glucoside; ○, 3-*O*-methylglucose; ■, Mn²⁺; □, Ca²⁺.

Results and Discussion

Effect of virus on accumulation of substances that enter by facilitated transport

Cells are able to accumulate sugars and other biosynthetic precursors present in extracellular fluid at low concentration by a facilitated uptake mechanism, followed by phosphorylation to yield a product that does not diffuse out of cells. In the case of amino acids, retention is achieved by coupling to the Na⁺ gradient across cells (e.g. Christensen, 1975). When virus is added to cells, there is a decrease in the uptake of such substances. As the concentration of substrate is increased, a 'crossover point' is reached, beyond which the effect is reversed (Fig. 1*a*).

It thus seems that, at low concentration of substrate, virus causes a failure to retain accumulated substrate and its metabolites. In the case of choline and deoxyglucose, substrate entering Lettrée cells is rapidly phosphorylated (Pasternak & Micklem, 1973) so that after a brief exposure most is present as phosphocholine and 2-deoxyglucose 6-phosphate respectively (Table 1); further metabolism is minimal in either case. (Longer exposure to radioactive choline, of course, labels phospholipids and other products.) Note that virus does not affect the degree of phosphorylation of 2-deoxyglucose at 10 μM, despite a fall in intracellular ATP content, as documented below (Table 5). Since it is known that

virus causes cells to become leaky to phosphorylated compounds (Pasternak & Micklem, 1973, 1974*a*), the effect on uptake of substances at low concentration is likely to be due to this cause.

That this is indeed so, and that the effect of virus is not on the transport mechanism itself, receives support from two types of experiment. First, the data of Table 1 show that phosphorylated metabolites already appear in the medium during a 5 min 'uptake' pulse; moreover, the total amount of phosphorylated compounds (that in cells plus that in medium) is the same in the presence of virus as in its absence (Table 1). In other words, the total amount of choline or 2-deoxyglucose passing into the cell is the same; it is its subsequent fate that is modified by virus. Second, the effect of virus on the uptake of 3-*O*-methylglucose, which is taken up by facilitated transport but is not phosphorylated (Le Fevre, 1961), was measured. Fig. 1(*b*) shows that the effect of virus is to increase, not to decrease, uptake even at low concentration; in other words the effect is similar to that on a substrate such as α-methyl glucoside (Pasternak & Micklem, 1974*b*; Fig. 3) or bivalent cations (Impraim *et al.*, 1979), which enter cells by passive diffusion alone (Fig. 1*b*). With bivalent cations, the viral effect is diminished at high concentration; this is because bivalent cations decrease virally induced permeability, including their own.

For amino acids at low concentration, the effect

Table 1. *Effect of virus on phosphorylated metabolites of choline and 2-deoxyglucose*
Lettrée cells (10⁸/ml) were exposed to labelled substrate with or without virus (250 HAU/ml) at 37°C for 5 min, spun through oil, and the medium and cell pellet were analysed as described in the Materials and Methods section. The radioactivity in the medium at the start of incubation was as follows: 10 μM-choline, 1.9 × 10⁶ c.p.m./ml; 20 mM-choline, 1.9 × 10⁶ c.p.m./ml; 10 μM-deoxyglucose, 1.8 × 10⁶ c.p.m./ml; 20 mM-deoxyglucose, 2.1 × 10⁶ c.p.m./ml. The percentage distribution of radioisotope in the various cell fractions is shown.

	Low concentration (10 μM)		High concentration (20 mM)	
	–virus	+virus	–virus	+virus
[¹⁴ C]Choline				
Medium				
Choline	59	74	96	93
Phosphocholine	3	11	<1	<1
Cells: water extract				
Choline	7	8	3	6
Phosphocholine	30	6	1	<1
Cells: residue	2	1	<1	<1
2-Deoxy[³ H]glucose				
Medium				
Deoxyglucose	24	21	90	88
Deoxyglucose 6-phosphate	9	60	<1	1
Cells: water extract				
Deoxyglucose	8	3	7	10
Deoxyglucose 6-phosphate	57	16	2	1
Cells: residue	2	1	<1	<1

of virus is as shown in Table 2. That is, inhibition of uptake is in the order glycine and aminoisobutyrate > valine and leucine. This is also the order in which amino acid uptake is linked to the Na^+ pump, as assessed by replacing Na^+ by K^+ in the incubation medium. The similarity between the effect of virus, and the degree of Na^+ linkage, is particularly clear-cut with glycine, as illustrated in Fig. 2. In other words, the effect of virus is not so much on the

Fig. 2. Comparison between the effect of virus and of Na^+ depletion on glycine uptake

Lettree cells were incubated for 12 min at 37°C , with or without virus (200 HAU/ml) in Na^+ medium, or in K^+ medium, with [^{14}C]glycine at the concentrations indicated, and uptake was assessed by spinning through oil. The lowest concentration of glycine was $0.1\mu\text{M}$. $\square$, Percentage inhibition by virus; O, percentage inhibition by substituting K^+ medium for Na^+ medium.

uptake of the amino acid itself as on the Na^+ gradient, which is abolished by virus (this is documented, in relation to membrane potential, more fully below); whether the inhibition of valine and leucine uptake at low concentration is due to some residual coupling mechanism or is the result of failure to retain accumulated leucine is not at present clear. The effect of virus on amino acid uptake is thus analogous to the action of uncoupling agents on H^+ -linked ATP synthesis in mitochondria: by making the membrane leaky, the ability to carry out ion-driven processes (Na^+ -linked amino acid transport, or H^+ -linked ATP synthesis) is lost.

Choline uptake has been shown to be Na^+ -linked in nerve cells (Yamamura & Snyder, 1972), and to be inhibited by conditions that cause membrane depolarization (Sen & Cooper, 1978; Sen *et al.*, 1978). Hence it is possible that the effect of virus on choline uptake is due to the abolition of the Na^+ gradient, as well as to increased leakage of phosphocholine. However, in Lettree cells, choline uptake is not Na^+ -linked; on the contrary choline uptake is increased in K^+ (Na^+ -free) medium (Table 3). In other words, the effect of virus on choline uptake is due entirely to increased leakage of phosphocholine, and not to abolition of the Na^+ gradient.

The results presented above account satisfactorily for the observed decrease by virus in the extent of uptake of substances that accumulate in cells. Substances that do not accumulate in cells, or that enter by passive diffusion, are affected in the opposite way. In this case, the primary effect of virus is on rate, rather than extent; however, because virally treated cells swell (Knutton *et al.*, 1976) [unless prevented from so doing by the presence of osmotically active substances in the medium, as illustrated below (Table 6)], an increase in extent of

Table 2. Effect of virus on uptake of amino acids

Lettree cells ($4 \times 10^7/\text{ml}$) suspended in Na^+ or K^+ medium, were exposed to labelled amino acid with or without virus (250 HAU/ml) at 37°C for 10 min, spun through oil and the radioactivity of the pellet was assayed. Low concentration was $1\mu\text{M}$; high concentration was 30 mM (except for leucine, which was at 10 mM). The radioactivity in the medium at the start of incubation was as follows: $1\mu\text{M}$ -glycine, 1.6×10^5 c.p.m./ml; 30 mM-glycine, 3.6×10^5 c.p.m./ml; $1\mu\text{M}$ -aminoisobutyrate, 2.0×10^5 c.p.m./ml; 30 mM-aminoisobutyrate, 4.7×10^5 c.p.m./ml; $1\mu\text{M}$ -valine, 1.5×10^5 c.p.m./ml; 30 mM-valine, 2.5×10^5 c.p.m./ml; $1\mu\text{M}$ -leucine, 3.0×10^5 c.p.m./ml; 10 mM-leucine, 3.1×10^5 c.p.m./ml. The values quoted are the percentage of the radioactivity in the medium that is taken by the cells.

	Low concentration				High concentration			
	Na^+ medium		K^+ medium		Na^+ medium		K^+ medium	
	—virus	+virus	—virus	+virus	—virus	+virus	—virus	+virus
[^{14}C]Glycine	33	5.1	4.2	4.5	4.4	4.0	1.8	3.8
Amino[^{14}C]isobutyrate	35	5.3	11	5.5	3.3	4.2	1.8	4.0
[^3H]Valine	21	7.4	22	5.0	4.6	4.6	1.9	4.0
[^3H]Leucine	24	6.7	25	5.3	3.5	4.2	2.4	4.4

uptake, owing to an increase in intracellular volume, is observed in addition to an increase in rate (see Fig. 1 of Pasternak & Micklem, 1974b). α -Methyl glucoside, which is neither phosphorylated nor taken up by a specific transport mechanism, is an example (Fig. 3); 3-*O*-methylglucose and bivalent cations behave similarly (Fig. 1b).

For the same reason, entry of choline, 2-deoxyglucose or amino acids at high concentrations, when facilitated transport is saturated and has insufficient capacity to bring the intracellular concentration up to that of the medium, is stimulated by virus (Fig. 1a and Tables 1 and 2; see also Pasternak & Micklem, 1974b; Micklem & Pasternak, 1977). In addition,

Fig. 3. Effect of virus on uptake of α -methyl glucoside. Lettrée cells were incubated at 22°C with or without virus (250 HAU/ml) in the presence of 10 mM- α -methyl [14 C]glucoside, and uptake was assessed by spinning through oil. The values were corrected for the [3 H]inulin space as described by Imprim *et al.* (1979). O, Uptake without virus; □, uptake with virus.

treatments that inhibit facilitated diffusion and thus make transport rate-limiting, such as Na⁺-free medium in the case of aminoisobutyrate and glycine (Table 2), or low temperature in the case of most substances (Table 3), show the same reversal of viral action. Thus the uptake at 5°C of glycine and aminoisobutyrate at low concentration is increased by virus; this is so despite the fact that uptake is still partially Na⁺-linked (values in parentheses in Table 3). For uptake of choline and 2-deoxyglucose at low concentration and low temperature, reversal of the viral effect is not complete (in contrast with the situation at high concentration, at either temperature); nevertheless, the inhibitory action of virus is much decreased (Table 3).

In summary, the data of Tables 2 and 3 show that the virally induced increase in simple diffusion leads to effective equilibration between the inside and outside of cells. That is, in the presence of virus, the uptake of most substances at any concentration is approx. 3–5% of the amount present in the medium. This value presumably corresponds to the percentage intracellular water space of (virally swollen) Lettrée cells at a concentration of 4×10^7 /ml. Higher percentage uptakes, which are generally seen only in the absence of virus, indicate a net accumulation. The above explanations of the inverse effects of virus are diagrammatically summarized in Fig. 4.

Outcome of permeability changes

(a) *Membrane depolarization.* The membrane potential of cells may conveniently be assayed by the use of cyanine dyes (Waggoner, 1976). We have used 3,3'-dipropylloxadinecarbocyanine, employing the methodology of Philo & Eddy (1978), in which inhibitors are added to minimize effects owing to mitochondrial cation transport. In this system an

Table 3. Effect of temperature on virally mediated uptake changes

Lettrée cells (4×10^7 /ml) in Na⁺ medium were exposed to labelled substrate with or without virus (250 HAU/ml) at 37°C or 5°C for 10 min, spun through oil and the radioactivity in the pellet was assayed. Low concentration was 1 μ M; high concentration was 30 mM. The radioactivity in the medium at the start of incubation was as follows: 1 μ M-choline, 9.0×10^4 c.p.m./ml; 30 mM-choline, 9.6×10^4 c.p.m./ml; 1 μ M-deoxyglucose, 1.2×10^5 c.p.m./ml; 30 mM-deoxyglucose, 2.0×10^5 c.p.m./ml; 1 μ M-glycine, 2.2×10^5 c.p.m./ml; 30 mM-glycine, 2.0×10^5 c.p.m./ml; 1 μ M-aminoisobutyrate, 1.5×10^5 c.p.m./ml; 30 mM-aminoisobutyrate, 3.2×10^5 c.p.m./ml. The values quoted are the percentage of the radioactivity in the medium that is taken up by the cells. Values in parentheses refer to uptake in K⁺ medium.

	Low concentration				High concentration			
	37°C		5°C		37°C		5°C	
	–virus	+virus	–virus	+virus	–virus	+virus	–virus	+virus
[3 H]Choline	56 (82)	6.5 (9.2)	8.1	7.6	2.7	5.4	2.3	6.8
2-Deoxy[3 H]glucose	65 (77)	13 (14)	45	11	3.4	5.2	3.3	4.2
[14 C]Glycine	19 (3.1)	4.9 (4.9)	2.5 (1.1)	5.3 (2.7)	6.1	3.9	0.8	5.0
Amino[14 C]isobutyrate	31 (12)	4.7 (6.1)	2.2	5.3	3.3	4.4	0.9	5.7

(a) Substances accumulated by phosphorylation

- (i) Low concentration: uptake by facilitated transport.
Effect of virus: lower amount in cells, owing to leakage of metabolites.

- (ii) High concentration: uptake predominantly by simple diffusion.
Effect of virus: increased rate of entry (and exit); increased amount in cells owing to swelling.

Note: dashed arrows indicate rate-limiting processes. The exit of substances has been omitted for clarity. The effects shown are for 37 °C; at 5 °C, the effect on substances at low concentration approaches that on substances at high concentration.

(b) Substances accumulated by linkage to Na⁺ gradient

- (i) Low concentration: uptake by facilitated mechanism (coupling to Na⁺ gradient).

Effect of virus: lower amount in cells, owing to abolition of Na⁺ gradient resulting from inward leakage of Na⁺.

- (ii) High concentration: uptake predominantly by simple diffusion.

Effect of virus: increased rate of entry (and exit); increased amount in cells owing to swelling.

Note: dashed arrows indicate rate-limiting processes. The movement of substances by exchange diffusion has been omitted for clarity. The effects shown are for 36 °C, in Na⁺ medium; at 5 °C, or in K⁺ medium, the effect on substances at low concentration resembles that on substances at high concentration.

Fig. 4. Summary of viral effects on uptake of substances at low and high concentration

increase in fluorescence indicates a decrease in membrane potential, and vice versa. Thus K⁺ added to cells results in an increase in fluorescence (depolarization), whereas valinomycin (in absence of K⁺) results in a decrease in fluorescence (hyperpolarization) (Fig. 5).

When virus is added to cells at 37 °C, the fluorescence begins to increase within 30 s, indicative of depolarization: the fact that fluorescence increases to an extent approximately equal to the fluorescence in the absence of the cells, and that

subsequent addition of valinomycin (in presence of K⁺) causes no further increase, suggests that the depolarization is virtually complete (i.e. the membrane potential is approximately zero). The effect is temperature-dependent, is inhibited by Ca²⁺, and resembles the permeability changes elicited by virus in other respects also (Table 4). The time at which Ca²⁺ is added is important: if added before, or less than 1 min after, virus, depolarization is prevented; if added approx. 1 min after depolarization has begun, further depolarization is prevented and repolarization

Fig. 5. Effect of virus on membrane potential

Lettree cells ($10^7/\text{ml}$) suspended in Na^+ medium were added to dye at the point indicated by $\downarrow$ and fluorescence intensity was measured as described in the Materials and Methods section. Concentrations of added substances refer to the final value. Measurements were made at 37°C , except as indicated below. (a) 66 mM-KCl (K) added as indicated. (b) Valinomycin (val; $13\text{ }\mu\text{g/ml}$) added as indicated, followed by 66 mM- and 132 mM-KCl (K) at the times indicated. (c) Virus (v; 40 HAU/ml) added as indicated. (d) As (c), but at 30°C . (e) As (c), but at 20°C . (f) Virus (v; 40 HAU/ml) added as indicated, followed by valinomycin (val; $13\text{ }\mu\text{g/ml}$). (g) Heat-killed virus (v; originally 40 HAU/ml) added as indicated. (h) Concanavalin A (Con A; $36\text{ }\mu\text{g/ml}$), followed by virus (v; 40 HAU/ml), added as indicated. (i) '1-day' virus (v; 30 HAU/ml) added as indicated. (j) '1-day' virus after freezing and thawing 10 times (v; 30 HAU/ml) added as indicated. (k)–(n) 5 mM-CaCl_2 (Ca) and virus (v; 40 HAU/ml) added as indicated. Time scale is indicated by the bars.

Table 4. Comparison between virally mediated permeability changes and membrane depolarization

	Permeability change	Membrane depolarization
Temperature at which change becomes significant	$>15\text{--}20^\circ\text{C}$ (Pasternak & Micklem, 1973)	$>20^\circ\text{C}$ (Fig. 5)
Effect of Ca^{2+}	Inhibition (Pasternak & Micklem, 1974a)	Inhibition (Fig. 5)
Effect of concanavalin A	Inhibition (Micklem & Pasternak, 1977)	Inhibition (Fig. 5)
'1-day' virus instead of '3-day' virus	No change (Impraim <i>et al.</i> , 1979)	No change (Fig. 5)
Freeze–thawed '1-day' virus	Change initiated (C. C. Impraim & C. A. Pasternak, unpublished work)	Change initiated (Fig. 5)

zation occurs. In other words the action of Ca^{2+} on permeability is instantaneous, in contrast with its action in re-establishing the Na^+ gradient, as reflected in aminoisobutyrate uptake (see Fig. 7 below), for example. The ouabain-sensitive ($\text{Na}^+ + \text{K}^+$) ATPase, which is responsible for establishing and maintaining the gradient, is not itself affected by virus (Poste & Pasternak, 1978); the supply of ATP, on the other hand, is (Table 5). Within 5 min of the addition of virus, for example, ATP concentrations have fallen by over 90%; clearly the time taken to re-establish a net Na^+ gradient reflects the time taken to replenish the ATP pool.

Note that virus causes a net loss of the intracellular adenine nucleotide pool in general, since AMP, like phosphocholine or 2-deoxyglucose 6-phosphate, leaks out of cells. ADP also appears to leak out of cells, but ATP does not (Table 5). It has, however, to be borne in mind that much of the ATP is likely to be mitochondrial, and it is the plasma membrane, not the mitochondrial membrane, that is modified by virus in the first instance. The reason for

the loss of ATP is not clear. Presumably as ATP is used up to pump out Na^+ (unsuccessfully in the face of the increased permeability of cells), ADP so formed is converted back into ATP (and AMP) by adenylate kinase; the net result is a conversion of ATP and ADP into AMP, which leaks out. The decrease in P_i through leakage out of cells (Table 5), may be another contributing factor to breakdown of adenine nucleotides (cf. Van den Berghe *et al.*, 1977).

(b) *Haemolysis*. A characteristic feature of paramyxoviruses is their ability to cause haemolysis. Unlike bacterially mediated haemolysis, enzymes such as phospholipase A do not appear to be responsible, since phospholipid degradation is not observed during attack by paramyxoviruses (Pasternak & Micklem, 1974a; Poste & Pasternak, 1978). Nor is the action of neuraminidase (Klemperer, 1960a) likely to be responsible (Micklem & Pasternak, 1977). Instead, haemolysis appears to result directly from virally mediated permeability changes; that is, the colloid osmotic pressure generated by the leakage of cations causes cells to

Table 5. *Effect of virus on metabolites of adenosine and P_i*

Lettrée cells were incubated at 37°C with [^{14}C]adenosine for 1 h or with $^{32}\text{P}_i$ for 30 min, washed, resuspended ($2 \times 10^8/\text{ml}$) and incubated with or without virus (150 HAU/ml) at 37°C for 5 min, spun through oil, and the medium and cell pellet were analysed as described in the Materials and Methods section. The percentage distribution of radioisotope in the various cell fractions is shown.

		Distribution (%)	
		–virus	+virus
^{14}C Adenosine-labelled cells	Medium		
	Adenosine		1
	AMP	6	52
	ADP		3
	ATP		<1
Cells: water extract or HClO_4 -soluble	Adenosine	4	1
	AMP	23	14
	ADP	13	6
	ATP	18	1
Cells: residue or HClO_4 -insoluble		35	22
		Distribution (%)	
		–virus	+virus
$^{32}\text{P}_i$ -labelled cells	Area*		
	Medium		
	P_i	20	62
	AMP	<0.2	1.5
	ADP	<0.2	1.3
Cells: HClO_4 -soluble	ATP	<0.2	0.5
	P_i	56	10
	AMP	1.2	4
	ADP	1.1	0.6
Cells: HClO_4 -insoluble	ATP	1.6	1.4
		20	20

* The distribution of ^{32}P in areas corresponding to P_i , AMP, ADP and ATP markers is quoted, and includes any substances co-migrating with these markers.

swell (Klemperer, 1960*b*). In the case of cells covered with microvilli or other protuberances, the latter can be ‘unfolded’ to give an increased volume (Knutton *et al.*, 1976); in the case of erythrocytes, only a partial increase (from biconcave disc to sphere) is possible, beyond which lysis ensues. Thus erythrocytes leak haemoglobin and other proteins (Klemperer, 1960*b*), whereas villated cells retain their protein (Pasternak & Micklem, 1973; Poste & Pasternak, 1978) and remain impermeable to Trypan Blue (Pasternak & Micklem, 1973). If haemolysis is indeed a direct consequence of permeability-induced osmotic swelling, then agents that (a) inhibit the permeability change or (b) prevent osmotic swelling should abolish haemolysis. Ca²⁺, which has long been known to inhibit haemolysis (Burnet & Lind, 1950; Fukai & Suzuki, 1955; Sato, 1958; Klemperer, 1960*b*, Neurath, 1965), is an agent of the first category (Pasternak & Micklem, 1974*a*); high concentrations of osmotically active substances such as glycerol, or substitution of KCl for NaCl as suspending medium, are agents of the

second category. Both types of agent inhibit haemolysis (Table 6). Another reason for assuming that haemolysis is the direct outcome of permeability changes is the following. Homma *et al.* (1976) have shown the Sendai virus harvested after growth in embryonated eggs for 1 day instead of 3 days (‘1-day’ virus) is non-haemolytic, yet infective; freeze–thawing such virus renders it haemolytic. We confirm this observation and show (Table 6, and Impraim *et al.*, 1979) that 1-day virus does not cause permeability changes, but that freeze–thawed 1-day virus does.

(c) *Giant-cell (polykaryon) formation.* A characteristic property of paramyxoviruses is their ability to cause cells to fuse together. That this property is, like haemolysis, a direct consequence of permeability-induced osmotic swelling is documented below. Note that we avoid use of the more usual term ‘cell–cell fusion’. This is because cell–cell fusion has already occurred by stage 2 (Fig. 6), as shown by electron microscopy (Knutton, 1979), though by

Fig. 6. Events leading to giant-cell (polykaryon) formation
For further details see the text.

Table 6. *Inhibitors of haemolysis and giant-cell formation*

A monolayer of human erythrocytes on collagen-coated plastic Petri dishes were exposed to virus (500HAU/ml) and fusion index measured as described by Bachi *et al.* (1978); haemolysis was assessed by the *A*₅₄₀ of released haemoglobin. Lettrée cells were treated with virus and the volume, permeability change and fusion index measured as described by Knutton & Pasternak (1979).

Treatment	Erythrocytes		Lettrée cells		
	Haemolysis (% of lysis by water)	Fusion index	Volume (μm ³)	Permeability change (% leakage)	Fusion index
None	<1	<0.01	540	8	<0.001
Virus	98	0.4	780	80	0.6
Virus + 0.38 M-glucose			540	75	<0.001
K ⁺ medium + virus	101	<0.01	760	78	<0.001
Virus + 1.2 M-glycerol	42				
Virus + 2.3 M-glycerol	7	<0.01			
Virus + 1 mM-CaCl ₂	41		730	55	0.6
Virus + 10 mM-CaCl ₂	1	<0.01	600	10	
‘1-day’ virus	5	0.08		15	
Freeze–thawed ‘1-day’ virus	90	0.4		75	

the criterion of light microscopy, used by other workers, cells at stage 2 appear 'unfused'.

Agents that prevent osmotic swelling, such as high concentrations of sugars or glycerol, or substitution of KCl for NaCl as suspending medium, prevent giant-cell formation (Table 6). Virally induced membrane permeability (as assessed by leakage of intracellular phosphorylated compounds) is not affected under these conditions, indicating that it is the net entry of water, consequent to a leakage of Na^+ and K^+ , that is required for giant-cell formation. Of course, if the change in permeability that is responsible for the abolition of the Na^+/K^+ asymmetry and the consequent entry of water is itself prevented, giant-cell formation does not occur either: the use of '1-day' virus instead of '3-day' virus, or the presence of a high concentration of Ca^{2+} , are examples (Table 6). Note that the concentration of Ca^{2+} necessary to prevent permeability changes is partly dependent on the amount of virus added; at low concentrations of cells and virus (at which agglutination, and hence giant-cell formation, does not occur) 0.1–0.5 mM- Ca^{2+} inhibits the permeability change by 50% (Pasternak & Micklem, 1974a; Impraim *et al.*, 1979). At the high concentrations of cells and virus required for cell–cell fusion, 1–2 mM- Ca^{2+} is insufficient. In fact some Ca^{2+} is probably necessary for the formation and stability of fused cells, since EDTA prevents the process (Pasternak & Micklem, 1974a).

The events leading to giant-cell formation may be summarized (Knutton & Pasternak, 1979) as follows (Fig. 6). First, cells are brought close together by agglutination. This is temperature-independent, since it occurs at 0°C as well as at 37°C. Next, the membranes between virus and cells fuse (virus–cell fusion); fusion requires a temperature greater than 15–20°C (Okada *et al.*, 1975; see also Pasternak & Micklem, 1973). Where a virus particle fuses simultaneously with two cells, a cytoplasmic bridge is formed (Apostolov & Almeida, 1972; Knutton, 1978). Cells in this 'dumb-bell' shape then expand, as a result of osmotic swelling, to form a giant cell. The evidence that membrane fusion precedes swelling, that is, that stage 2 (Fig. 6) is a distinct entity, is two-fold. First, cells suspended in KCl do not form giant cells (Table 6); although such cells swell, they do so before the addition of virus; hence the 'driving force' for converting stage 2 into stage 3 is lost. Second, cells treated with '1-day' virus become arrested at stage 2 (Knutton, 1979), because '1-day' virus does not cause permeability changes; if such cells are exposed to hypoosmotic medium, giant-cell formation ensues (Knutton & Pasternak, 1979).

Recovery of cells

The above has shown that changes that occur in the cytoplasm of cells exposed to Sendai virus can be

ascribed to an increase in non-specific membrane permeability. Recovery of cells is dependent on restoration of the original permeability barrier. This can be achieved in one of two ways: by Ca^{2+} , which temporarily restores the permeability barrier, and by incubation at 37°C in growth medium, during which time the surface membrane is gradually repaired.

The action of Ca^{2+} is almost instantaneous insofar as restoration of the permeability barrier to ions (Fig. 5), phosphorylated compounds (see Fig. 8), and other compounds that cross the plasma membrane by passive diffusion, is concerned (Fig. 7). Restoration of aminoisobutyrate uptake takes longer (over 70 min in the experiment illustrated in

Fig. 7. Reversal of virally mediated changes by Ca^{2+} (a) Uptake of amino[^{14}C]isobutyrate (upper graph) and [^3H]choline (lower graph). Lettrée cells ($3 \times 10^7/\text{ml}$) were exposed to a 3 min pulse of $10 \mu\text{M}$ -amino[^{14}C]isobutyrate and 20 mM -[^3H]choline at 37°C at the times indicated, and uptake was measured by spinning through oil. Virus ($20 \text{ HAU}/\text{ml}$) and CaCl_2 (5 mM) were added at the times indicated. ●, Without virus or Ca^{2+} ; □, with virus (v); ○, with virus plus Ca^{2+} (Ca). (b) Leakage of [^3H]phosphocholine. Lettrée cells were incubated with [^3H]choline, exposed to virus ($5 \times 10^{-5} \text{ HAU}/\text{cell}$) in the presence of CaCl_2 (0.5 mM), and release of ^3H was measured by the continuous-flow technique described by Impraim *et al.* (1979). ■, No additions; ▲, EDTA (1 mM) added as indicated; ●, CaCl_2 (0.5 mM) added as indicated; a further addition of EDTA (1 mM) was made as indicated.

Fig. 7), presumably because restoration of the Na^+ gradient, through operation of the Na^+ pump, takes time; restoration of the Na^+ gradient is insensitive to inhibition of protein synthesis. The observation that restoration of the membrane potential by Ca^{2+} is achieved within 1 min (Fig. 5) reflects the fact that membrane potential and Na^+ gradient are separate parameters: during a single depolarization of a neuron, for example, the overall Na^+ gradient is maintained despite reversal of the membrane potential, and conversely virally modified cells, in which the Na^+ gradient has been lost through equilibration between internal and external Na^+ , may yet show

normal membrane potential [compare Fig. 7(a) with Fig. 5, traces (l)–(n)].

Recovery of cells by the action of Ca^{2+} is temporary in the sense that, if the Ca^{2+} is subsequently removed by chelation with EGTA, the leaky nature of the cell membrane is partially restored (Figs. 7b and 8); washing cells has the same effect. Recovery of cells by incubation at 37°C is permanent in the sense that the original permeability barrier is completely restored. Recovery takes about 2–4 h for HeLa cells (Poste & Pasternak, 1978). This probably reflects the turnover time of plasma membrane (Tweto & Doyle, 1977); dispersion of viral components by diffusion in the plane of the membrane is quite rapid (Bachi *et al.*, 1973) and therefore appears to be insufficient to restore the membrane to its normal state. Membrane turnover can occur through recycling of existing membrane and, like restoration of the Na^+ gradient, is insensitive to inhibitors of protein synthesis (J. Duncan, K. J. Micklem & C. A. Pasternak, unpublished work). Recovery is temperature-dependent (see Table 2 of Impraim *et al.*, 1979). If cells are kept at 4°C , the viral effect is variable and depends on factors such as the original viral dose, the presence of Ca^{2+} , the constituents of the medium, and so forth. What is clear is that at 37°C the maximum effect of virus can be quite short-lived. Fig. 9 shows this in the case of passive uptake of $[^3\text{H}]$ choline; leakage of $[^3\text{H}]$ choline metabolites is illustrated in Fig. 7 of Impraim *et al.* (1979).

Fig. 8. *Reversal of Ca^{2+} -mediated recovery by EGTA* (a) Leakage of phosphocholine. Lettrée cells were incubated with $[^3\text{H}]$ choline, washed and resuspended in medium containing 1 mM- CaCl_2 . Virus (130 HAU/ml) was added and cells were incubated at 37°C . At 0 and 9 min after the start of incubation, a mixture of EGTA and CaCl_2 was added to bring the concentration to 4.4 mM-EGTA, 3.2 mM- CaCl_2 . Samples were analysed at intervals and the distribution of ^3H between cells and medium was assessed as described in the Materials and Methods section. O, No addition; ■ and □, EGTA/ CaCl_2 added at times indicated. (b) Uptake of 15 mM-choline. Lettrée cells (3×10^7 /ml) were incubated with 15 mM- $[^3\text{H}]$ choline with or without 6 mM- CaCl_2 (final concn.) and with or without virus (250 HAU/ml), and uptake was measured by spinning through oil. O, Without virus; ●, without virus, in 6 mM- Ca^{2+} ; □, with virus, in 6 mM- Ca^{2+} ; ■, with virus, in 6 mM- Ca^{2+} , with EGTA (14 mM) added at 0 or 10 min respectively.

Fig. 9. *Effect of temperature on duration of virally mediated permeability changes*

Lettrée cells (10^8 /ml) were incubated with or without virus (500 HAU/ml) for 5 min at 37°C . They were then either maintained at 37°C or chilled to 5°C . At the times indicated, suspensions were exposed to 20 mM- $[^3\text{H}]$ choline for 3 min at 5°C and uptake was measured by washing in buffer. O, Without virus, at 37°C ; ●, without virus at 5°C ; □, with virus, at 37°C ; ■, with virus, at 5°C .

Conclusion

The results presented in this and in previous papers, and summarized by Knutton (1978), Poste & Pasternak (1978) and Knutton & Pasternak (1979), may be diagrammatically represented as shown in Fig. 10.

It is clear that the permeability changes are not a prerequisite for viral entry into cells. Any clinical significance is therefore not immediately obvious, especially as the changes are prevented by Ca^{2+} at concentrations of Ca^{2+} near those of plasma. Nevertheless virally mediated permeability changes may well contribute to the pathology of viral infections, for, at high virus concentrations, 2 mM- Ca^{2+} is ineffective at inhibiting the permeability change (Pasternak & Micklem, 1974a); 2 mM- Ca^{2+} is also ineffective at inhibiting the change in certain organ cultures (K. A. Foster & C. A. Pasternak, unpublished work). In any case, the concentration of Ca^{2+} in interstitial fluid is less than that of plasma; also, fluctuation in Ca^{2+} concentrations may occur in certain disease states. Hence the possibility that permeability changes, especially with regard to Na^+ and K^+ movements and membrane depolarization (Fig. 5), contribute to some of the consequences of viral infection such as encephalopathy (Illis, 1975) or intestinal disorders (Kerzner *et al.*, 1977) warrants serious consideration.

So far as haemolysis (Table 6) is concerned, it is unlikely to be of much clinical relevance. Most types of haemolytic anaemia that are associated with viral infections are the result of an auto-immunity, rather than of a direct haemolytic effect of the virus (e.g. Dacie, 1963), though the generation of auto-antibodies possibly involves an initial haemolytic event. Destruction of blood platelets (thrombocytopenia), on the other hand, may be an outcome of direct viral action in a number of cases (Wintrobe, 1974); in this instance the underlying mechanism is not known.

Characterization of virally mediated changes is of importance in areas other than those of clinical medicine. The first relates to the properties of

communicating ('gap') junctions, for the virally mediated permeability channel resembles that created by a communicating junction (Micklem & Pasternak, 1977) in several regards. Phosphorylated compounds such as nucleotides and sugar phosphates, and ions such as Na^+ and K^+ , all of which leak only slightly out of cells under normal conditions, pass freely into the medium (virally modified cells) or from cell to cell (communicating junction). High-molecular-weight compounds, such as proteins or nucleic acids, do not pass through either type of channel (Pitts, 1976; Poste & Pasternak, 1978). Each channel is 'closed' by Ca^{2+} (Rose & Loewenstein, 1975; Pasternak & Micklem, 1974a; Loewenstein, 1979). Virally induced channels may thus be considered as a kind of 'half' communicating junction. One difference between the two types of channel lies in the nature of the intramembrane particles revealed by freeze-fracture; virally induced channels show no obvious changes in intramembrane particles (Knutton, 1977), whereas in communicating junctions intramembrane particles form a distinct pattern of aggregates (Peracchia, 1977). Since the mechanism by which Ca^{2+} regulates the flow of compounds through communicating junctions is not yet clear, information about the action of Ca^{2+} in the rather simpler virus-cell system may prove to be of use.

The second situation concerns the permeability properties of transformed cells in culture. These show increased leakage of phosphorylated compounds (nucleotides and 2-deoxyglucose 6-phosphate) and of univalent cations such as Rb^+ (an analogue of K^+) when external ATP is raised (Rozengurt *et al.*, 1977), or internal ATP is lowered (Rozengurt & Heppel, 1979); as in virally mediated permeability changes, the effect does not occur below 20°C, and proteins such as lactate dehydrogenase do not leak out (Rozengurt *et al.*, 1977). As yet, the effects of Ca^{2+} have not been fully studied in this situation, though there is an indication that it may be inhibitory to leakage

Fig. 10. Significance of virally mediated permeability changes
For further details see the text.

Rozengurt *et al.*, 1977), and this would certainly be expected by analogy with virally mediated changes. Since ATP concentrations fall rapidly after the addition of virus (Table 5), it may be wondered whether virally mediated changes are a consequence of such a decrease in cellular ATP. This is unlikely. First, virally mediated changes occur as well in 3T3 cells as in SV40/3T3 cells (Poste & Pasternak, 1978), and this is not true of changes triggered by external ATP (Rozengurt *et al.*, 1977). Second, all the characteristics of virally mediated changes suggest that the decrease in ATP is a consequence of increased permeability, not vice versa. On the contrary, it is possible that the inhibitors used by Rozengurt & Heppel (1979) potentiate the permeability change by an action on the plasma membrane that is a cause (rather than a consequence) of the decrease in intracellular ATP.

Third, the effect of virus on the uptake of some amino acids confirms the electrogenic nature of that process. Just as the omission of Na^+ , or the addition of ouabain, gives an indication of the extent to which uptake is coupled to the movement of Na^+ , so does the addition of Sendai virus; because virus leads to an inverse effect on uptake of, for example, glycine in Na^+ or K^+ medium (Tables 2 and 3), electrogenic coupling is indicated in a particularly clear-cut way (Fig. 2). Moreover, with virus, Na^+ -dependence can be revealed in cells that are in contact with normal Na^+ medium. The effect on other amino acids, and indeed on other compounds, should prove rewarding in elucidating the extent to which the entry and accumulation of such compounds is linked to the Na^+ gradient.

Finally, the increased permeability induced in cultured cells by Sendai virus is useful for the introduction into cells of compounds that do not normally cross the plasma membrane. In studying the intracellular mechanism by which cellular growth is controlled, for example, it is desirable to be able to introduce cyclic nucleotides and other phosphorylated compounds. The exposure to a hyperosmotic medium (Castellot *et al.*, 1978) and to lysophosphatidylcholine (Miller *et al.*, 1979) have been suggested; Sendai virus (u.v.-inactivated to render it non-infectious) is a useful alternative. It too allows entry of phosphorylated compounds to take place and, as demonstrated in this paper, has the advantage that the extent and duration of the permeability change can be effectively controlled by Ca^{2+} . Moreover the size of pore is probably smaller, since Trypan Blue is excluded (Pasternak & Micklem, 1973), whereas it is taken up by the treatments of Castellot *et al.* (1978). The use of Sendai virus at exceptionally high virus:cell dose probably results in even bigger pores, and has been used to introduce macromolecules into cells (Tanaka *et al.*, 1975; Yamaizumi *et al.*, 1979).

We are grateful to the Science Research Council, Roche Products and the Ghana Atomic Energy Commission for financial support.

References

- Apostolov, K. & Almeida, J. D. (1972) *J. Gen. Virol.* **15**, 227–234
- Bachi, T., Aguet, M. & Howe, C. (1973) *J. Virol.* **11**, 1004–1012
- Bachi, T., Eichenberger, G. & Hauri, H. P. (1978) *Virology* **85**, 518–530
- Burnet, F. M. & Lind, P. E. (1950) *Aust. J. Exp. Biol. Med. Sci.* **28**, 129–150
- Castellot, J. J., Miller, M. R. & Pardee, A. B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 351–355
- Christensen, H. N. (1975) *Curr. Top. Membr. Transp.* **6**, 227–258
- Dacie, J. V. (1963) *The Haemolytic Anaemias*, 2nd edn., part II, p. 587, J. A. Churchill, London
- Fuchs, P. & Giberan, E. (1973) *FEBS Lett.* **31**, 127–130
- Fukai, K. & Suzuki, T. (1955) *Med. J. Osaka Univ.* **6**, 1–15
- Harris, H. (1970) *Cell Fusion*, Clarendon Press, Oxford
- Homma, M., Shimizu, K., Shimizu, Y. K. & Ishida, N. (1976) *Virology* **71**, 41–47
- Illis, L. S. (1975) *Viral Diseases of the Central Nervous System*, Balliere-Tindall, London
- Impraim, C. C., Micklem, K. J. & Pasternak, C. A. (1979) *Biochem. Pharmacol.* **28**, 1963–1969
- Kerzner, B., Kelly, M. N., Gall, D. G., Butler, D. G. & Hamilton, J. R. (1977) *Gastroenterology* **72**, 457–462
- Klemperer, H. G. (1960a) *Virology* **12**, 495–498
- Klemperer, H. G. (1960b) *Virology* **12**, 540–552
- Knutton, S. (1977) *J. Cell Sci.* **28**, 189–210
- Knutton, S. (1978) *Micron* **9**, 133–154
- Knutton, S. (1979) *J. Cell Sci.* **36**, 85–96
- Knutton, S. & Pasternak, C. A. (1979) *Trends Biochem. Sci.* **4**, 220–223
- Knutton, S., Jackson, D., Graham, J. M., Micklem, K. J. & Pasternak, C. A. (1976) *Nature (London)* **262**, 52–54
- Le Fevre, P. G. (1961) *Pharmacol. Rev.* **13**, 39–70
- Loewenstein, W. R. (1979) *Biochim. Biophys. Acta* **560**, 1–65
- Martin, S. J. (1978) *The Biochemistry of Viruses*, p. 28, Cambridge University Press, Cambridge
- Micklem, K. J. & Pasternak, C. A. (1977) *Biochem. J.* **162**, 405–410
- Miller, M. R., Castellot, J. J. & Pardee, A. B. (1979) *Exp. Cell Res.* **120**, 421–425
- Neurath, A. R. (1965) *Acta Virol. (Engl. Ed.)* **9**, 119–129
- Okada, Y. (1958) *Biken's J.* **1**, 103–110
- Okada, Y., Koseki, I., Kim, J., Maeda, Y., Hashimoto, T., Kanno, Y. & Matsui, Y. (1975) *Exp. Cell Res.* **93**, 368–378
- Pasternak, C. A. & Micklem, K. J. (1973) *J. Membr. Biol.* **14**, 293–303
- Pasternak, C. A. & Micklem, K. J. (1974a) *Biochem. J.* **140**, 405–411
- Pasternak, C. A. & Micklem, K. J. (1974b) *Biochem. J.* **144**, 593–595

- Pasternak, C. A., Strachan, E., Micklem, K. J. & Duncan, J. (1976) in *Cell Surfaces and Malignancy: Fogarty Int. Center Proc. 28* (Mora, P. ed.), pp. 147–151, U.S. Government Printing Office, Washington D.C.
- Peracchia, C. (1977) *Trends Biochem. Sci.* **2**, 26–31
- Philo, R. D. & Eddy, A. A. (1978) *Biochem. J.* **174**, 801–810
- Pitts, J. D. (1976) in *Developmental Biology of Plants and Animals* (Graham, C. F. & Wareing, P. F., eds.), pp. 96–110, Blackwell Scientific Publications, Oxford
- Poste, G. & Pasternak, C. A. (1978) *Cell Surf. Rev.* **5**, 306–349
- Rose, B. & Loewenstein, W. R. (1975) *Nature (London)* **254**, 250–252
- Rozengurt, E. & Heppel, L. A. (1979) *J. Biol. Chem.* **254**, 708–714
- Rozengurt, E., Heppel, L. A. & Friedburg, I. (1977) *J. Biol. Chem.* **252**, 4584–4590
- Sato, S. (1958) *Virusu* **8**, 48–58
- Sen, I. & Cooper, J. R. (1978) *J. Neurochem.* **30**, 1369–1375
- Sen, I., Baba, A., Schulz, R. A. & Cooper, J. R. (1978) *J. Neurochem.* **31**, 969–976
- Tanaka, K., Sekiguchi, M. & Okada, Y. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4071–4075
- Tweto, J. & Doyle, D. (1977) *Cell Surf. Rev.* **4**, 137–163
- Van den Berghe, G., Bronfman, M., Vanneste, R. & Hers, H. G. (1977) *Biochem. J.* **162**, 601–609
- Waggoner, A. (1976) *J. Membr. Biol.* **27**, 317–334
- Wintrobe, M. M. (1974) *Clinical Haematology*, 7th edn., p. 1101, Lea and Febiger, Philadelphia
- Yamaizumi, M., Uchida, T. & Okada, Y. (1979) *Virology* **95**, 218–221
- Yamamura, H. I. & Snyder, S. H. (1972) *Science* **178**, 626–628