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Effect of Vesicular Stomatitis Virus and Semliki Forest Virus on Uptake of Nutrients and Intracellular Cation Concentration

By M. A. GRAY,1 K. J. MICKLEM,1† F. BROWN2 AND C. A. PASTERNAK1*

1 Department of Biochemistry, St George's Hospital Medical School, London SW17 0RE and
2 Animal Virus Research Institute, Pirbright, Woking, Surrey GU24 0NF, U.K.

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SUMMARY

BHK-21 cells showed an increased ability to concentrate 2-deoxy-D-glucose (dGlc) 2 to 3 h after infection with vesicular stomatitis virus (VSV) or Semliki Forest virus (SFV), which began to be released at 2 and 3 h post-infection respectively; uptake of other nutrients was not affected in this way. Intracellular Na+ was either unchanged (VSV-infected cells) or increased (SFV-infected cells); K+ content was unchanged. These results do not support the current hypothesis that a non-specific increase in membrane permeability occurs in cells infected with rhabdoviruses or togaviruses.

INTRODUCTION

It is well established that stimulation of quiescent cells by mitogenic factors leads to an increased uptake of sugars and amino acids at an early stage in the sequence of events leading to an increased synthesis of DNA (for review, see Antoniades & Owen, 1982); cells transformed by oncogenic viruses (see Pasternak & Knox, 1977), and malignant as opposed to non-malignant cells (White et al., 1981), often show a similar increase. The infection of cells by cytolytic, non-transforming viruses such as vesicular stomatitis virus (VSV) or Semliki Forest virus (SFV) might be thought to have, if anything, an opposite effect on membrane function in that the synthesis of host DNA, RNA and protein becomes depressed (Pfefferkorn & Shapiro, 1974; Wagner, 1975), rather than elevated (but see Peluso et al., 1977). However, we show here that BHK cells infected with VSV or SFV accumulate 2-deoxy-D-glucose two to three times more effectively than uninfected cells.

Because of the suggestion (Carrasco, 1978, 1981) that non-specific membrane permeability is increased in cells infected with VSV or SFV we have measured other parameters, such as the uptake of amino acids and the intracellular concentration of monovalent cations, that might be expected to alter in 'leaky' cells. No evidence of a generalized increase in membrane permeability was apparent.

METHODS

Cells and virus. BHK-21 (clone 13) cells were infected with SFV at 4 × 107 p.f.u./plate (20 to 30 p.f.u./cell) for 1 h, washed and maintained in Dulbecco's minimal essential medium plus 2% newborn calf serum. The same cell line was infected with VSV (Indiana serotype) at 108 to 109 p.f.u./plate (10 to 100 p.f.u./cell). The release of VSV occurred faster than that of SFV, and hence samples were taken at earlier times post-infection. Because the uptake of compounds like amino-isobutyrate or deoxyglucose is particularly sensitive to changes in medium, cells were exposed to maintenance medium (RPMI 1640 plus L-glutamine, buffered to pH 7.4 with 1 M-HEPES instead of with NaHCO3) for 2 h before adding concentrated virus (or an equivalent amount of conditioned medium, i.e. medium derived from non-infected cells under the same conditions as medium containing virus), which was then left in contact with cells for the rest of the experiment. All work with VSV was carried out at the Animal Virus Research Institute, Pirbright, Surrey, so that the necessary disease security precautions could be met. Virus released into the medium was measured by conventional techniques: plaque assay on BHK-21 cells for all SFV experiments; plaque assay or LD50 assay in mice for VSV. The two latter methods gave identical results.

† Present address: Department of Biochemistry, University of Oxford, Oxford OX1 2JD, U.K.
Representative plates were taken at intervals throughout the period of study and stained with trypan blue. All results quoted in this paper refer to cells over 90% of which exclude trypan blue.

Uptake of labelled compounds. Uptake of radiolabelled compounds (all from Amersham International) was measured as follows. Cells in monolayer culture were washed and then exposed to \([3H]\)leucine (1 to 2 µCi/ml), \([2-3H]\)deoxy-d-glucose (dGlc; 1 to 5 µCi/ml), \([2-14C]\)amino-isobutyrate (AIB; 0.1 to 1 µCi/ml) or \([1-14C]\)aminocyclopentane-1-carboxylic acid (cycloleucine; 0.1 to 1 µCi/ml) in HEPES-buffered saline (HBS; Impraim et al., 1980; 0.5 ml for 35 mm diam. plates; 1 ml for 50 mm diam. plates) for various time periods. All operations were carried out in a hot room at 37 °C.

Radiolabel was removed by suction and the plates were washed rapidly four times in HBS or HEPES-buffered isotonic choline chloride (for experiments in which monovalent cations were measured), followed by addition of 0.25 or 0.5 ml of 0.1% Triton X-100 or 0.5 ml ice-cold 5% trichloroacetic acid (TCA); in the latter case plates were transferred rapidly to a refrigerator. The mixture of cells and extracting medium was transferred to microfuge tubes, centrifuged, and the supernatant used for assay of radiolabel and monovalent cations. All pellets were resuspended by brief sonication. Triton pellets were used directly for assay of DNA. TCA pellets were washed three times in ice-cold 5% TCA, and then used for assay of DNA, total protein or protein synthesis.

Cells were sampled over a minimum of three time points; for all values quoted (with the exception of leucine), uptake was linear over the time period studied (generally 1 to 10 min), and the values shown are means of the rate. With \([3H]\)leucine, uptake was usually maximal by 1 min and the values quoted, which are means of three or more determinations, represent amounts.

Results are shown as points on a time curve, with separate experiments indicated by different symbols. In each case, results are calculated per µg of DNA or protein, and expressed relative to the uptake by mock-infected cells taken as 100.

Other assays. Protein was measured by the method of Lowry et al. (1951) and DNA by the method of Cesarone et al. (1979). Water content was measured by scraping cells suspended in HBS into weighed microfuge tubes, centrifuging through oil, and weighing the cell pellet before and after overnight exposure to P₂O₅ desiccant. Monovalent cations were measured by atomic absorption.

RESULTS

VSV-infected cells

The time course of VSV replication in BHK-21 cells is shown in Fig. 1; protein synthesis began to decline at about the same time as virus production was maximal (Fig. 1 g). Uptake of leucine (expressed as TCA-soluble material) increased slightly during infection at a time when incorporation into TCA-insoluble material had diminished, but uptake of the non-metabolizable analogue cycloleucine was unaltered (Fig. 1 d); uptake of the non-metabolizable alanine analogue AIB decreased somewhat (Fig. 1 b). The most significant change, however, was in the uptake of dGlc which increased 1.5- to 2-fold (Fig. 1 a). Exposure of infected cells to a mixture of \([3H]\)dGlc and \([14C]\)AIB followed by measurement of the \(^3\)H : \(^14\)C ratio in washed cell extracts, provides a sensitive and diagnostic measure of the course of viral infection, which is independent of any cell loss that may occur. In each of the six preliminary experiments performed with VSV-infected BHK cells, in which there was variation in input virus, cell number, and other parameters as the methodology finally adopted (Methods) was being worked out, there was a consistent change in \(^3\)H : \(^14\)C ratio of infected cells relative to controls from 2 h post-infection onwards (Fig. 1 c). As seen from Fig. 1, the change in ratio occurred at the same time as VSV was released into the medium; it was also at this time that inhibition of protein synthesis became appreciable. The change in uptake ratio is thus a characteristically early event in cell surface function that precedes the subsequent inability to exclude trypan blue and other cytopathic changes (David-West & Osunkoya, 1971) by several hours; only the recently documented inhibition of pinocytosis (Wilcox et al., 1982) precedes it in time. The basis of these changes is as yet unclear; alterations in the pool size of endogenous compounds have not been ruled out. The changes are not due to re-infection by released virus, since VSV elicits no 'immediate' permeability changes (Foster et al., 1980).

Genty (1975) has found that uridine accumulation by VSV-infected embryonic chick cells is decreased. The onset of the effect is dependent on the dose of virus used: at an m.o.i. greater than 1, uptake declines from the end of the adsorption period (45 min) onwards; at multiplicities less than 1, onset of the change is delayed by several hours.
Fig. 1. VSV-infected BHK-21 cells. Cells in monolayer culture were infected with VSV and uptake of 10 µM[^3]H]dGlc (a), 10 µM[^14]C]AIB (b), 1-0 to 1-7 µM[^3]H]leucine (d, closed symbols) or 1-0 to 1-7 µM[^14]C]cycloleucine (d, open symbols) was measured as described in Methods; the ratio of[^3]H]dGlc:[^14]C]AIB is shown in (c). At the same time the content of K⁺ (e, closed symbols), Na⁺ (e, open symbols) and water (f, open symbols) was measured; the ratio of K⁺:Na⁺ is shown in (f) (closed symbols). Each separate experiment is indicated by a different symbol (circles, squares, triangles, etc). All values shown are for infected cells, expressed as a percentage of those for mock-infected cells sampled at the same time. The uptake (pmol/min/µg DNA) by mock-infected cells during the course of the experiment was as follows (% of pulse incorporated per plate in 10 min shown in parentheses): dGlc, 0-1 to 1-3 (2 to 10%); AIB, 0-1 to 0-3 (2 to 6%); leucine 0-7 to 4 (15 to 30%); cycloleucine 0-4 to 4 (13 to 27%). The content of cations and water of mock-infected cells during the course of the experiment was as follows: K⁺, 3 to 16 nmol/µg DNA; Na⁺, 0-6 to 3 nmol/µg DNA (K⁺:Na⁺ ratio 5-3 to 5-5); water, 20 to 100 nl/µg DNA. Virus released into the medium (LD₅₀ assay) is indicated in (g) (open symbols; solid line). The apparent rate of protein synthesis (calculated as % of total[^3]H]leucine that is TCA-precipitable and expressed as a percentage of that for mock-infected cells) is indicated in (g) (closed symbols); the rate in mock-infected cells was 2 to 8%/min.
Intracellular K⁺ and Na⁺ showed no significant change up to 4 h after infection (Fig. 1e); thereafter, intracellular K⁺ decreased and intracellular Na⁺ increased slightly, which is reflected in the K⁺/Na⁺ ratio (Fig. 1f). Francoeur & Stanners (1978) likewise found that VSV-infected L cells do not lose K⁺ before 5 h after infection. Water content of BHK-21 cells was unchanged over the first 4 h (Fig. 1f). Whatever the reason for the rounding-up and other cytopathic changes seen in VSV infections (Bablanian, 1975), entry of water or alteration of Na⁺/K⁺ content does not appear to be one of them. Nor do these results support the hypothesis that sensitivity of the protein-synthesizing system to hygromycin B in VSV-infected cells is due to membrane leakiness (Benedetto et al., 1980). Furthermore, it is clear that, in contrast to the hypothesis of Carrasco (1977), neither virus synthesis, which had reached a maximum by 4 h post-infection (Fig. 1g), nor inhibition of host protein synthesis (g), is dependent on an increase in intracellular Na⁺ (Fig. 1e); similar conclusions have been reached with regard to infection of cells by herpes simplex virus (Fenwick & Walker, 1978), poliovirus (Nair et al., 1979) or vaccinia virus (Norrie et al., 1982), although Garry et al. (1979, 1982) do find a temporal correlation between changes in intracellular cations and the switch-off of host protein synthesis in cells infected with VSV or Sindbis virus which they attribute to a decreased activity of the surface membrane Na⁺/K⁺-ATPase activity (Garry et al., 1982).

SFV-infected cells

Although SFV is a positive-strand RNA virus whereas VSV is negative-stranded, there is considerable similarity in their effect on cells. Not surprisingly, this is borne out in the present investigations of membrane function.

BHK-21 cells (the same clone was used for these experiments as for the VSV experiments) infected with SFV began to release virus at about 3 h post-infection (Fig. 2g). At this time total protein synthesis began to decline sharply (g). As with VSV-infected cells, AIB uptake decreased slightly, cycloleucine uptake was unaffected and leucine uptake (expressed as TCA-soluble material) increased slightly (Fig. 2d); as with VSV-infected cells, the increased amount of [³H]leucine in the TCA-soluble extract of infected cells is probably related to the inhibition of protein synthesis that is manifest at this time: total [³H]leucine in cells was unaltered. Again, the most significant change was an increase in the uptake of dGlc (Fig. 2a). The uptake of choline, which like dGlc accumulates in cells by an ATP-coupled phosphorylation mechanism (Impraim et al., 1980), did not increase, indicating that an increase in ATP availability is not the underlying mechanism for the increased dGlc accumulation. Moreover, preliminary results indicate that the uptake of 3-O-methylglucose, which is not phosphorylated, is also stimulated by SFV infection of BHK-21 cells.

The content of K⁺ (Fig. 2e) remained unchanged well past the 'cross-over' point (ratio of accumulated dGlc : AIB) which, as with VSV-infected cells, occurred at about the same time as virus release and inhibition of protein synthesis (Fig. 2c, g). Na⁺ content, on the other hand, appeared to increase rather early in infection (Fig. 2e). This increase did not appear to be matched by an equivalent decrease in K⁺ (Fig. 2f): a twofold increase in intracellular Na⁺ would correspond to a 20 to 30% decrease in K⁺, and this would have been detected. Hence, the increase in Na⁺ is not due to the sort of 'membrane leakiness' postulated to occur in SFV-infected cells (Carrasco, 1978). Whether the increase in intracellular Na⁺ contributes to the shut-off of host protein synthesis in this situation has yet to be investigated; certainly a much greater increase in intracellular Na⁺ brought about by treatment of BHK-21 cells with nigericin leads to a much smaller inhibition of protein synthesis than does infection with SFV (M. A. Gray, unpublished experiments).

Some of these changes, namely the increased uptake of dGlc, resemble those elicited by mitogens. Because mitogens are known to include cell-derived growth factors (Antoniades & Owen, 1982), and because virus preparations are by definition derived from cells, the possibility has to be considered that the effects are not due to an action of the virus itself. This is unlikely because uninfected cells were treated with conditioned medium, derived from cells in the same manner as the viral preparation. Moreover, heating the viral preparation at 56 °C for 1 h, or treating it with u.v. light for 1 h, reduced markedly the increased uptake of dGlc (Table 1) as well
Fig. 2. SFV-infected BHK-21 cells. Cells in monolayer culture were infected with SFV and uptake of labelled compounds, cation and water content, virus released into the medium, and apparent rate of protein synthesis measured and expressed exactly as described in the legend to Fig. 1. The relevant data for mock-infected cells are given in the legend to Fig. 1. (a) [\( ^3 \)H]dGlc uptake; (b) [\( ^{14} \)C]AIB uptake; (c) [\( ^3 \)H]dGlc:[\( ^{14} \)C]AIB ratio; (d) closed symbols, [\( ^3 \)H]leucine uptake and open symbols, [\( ^{14} \)C]cycloleucine uptake; (e) closed symbols, K\(^+\) content and open symbols, Na\(^+\) content; (f) closed symbols, K\(^+\)/Na\(^+\) ratio and open symbols, H\(_2\)O content; (g) closed symbols, apparent rate of protein synthesis and open symbols, virus released into medium.
Table 1. Effect of various treatments on dGlc uptake*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time post-infection (h)</th>
<th>dGlc uptake (infected as % of mock-infected)</th>
<th>Protein synthesis (treated as % of untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heating SFV for 1 h at 56 °C</td>
<td>2</td>
<td>124</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>4·5</td>
<td>277</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>6·5</td>
<td>269</td>
<td>192</td>
</tr>
<tr>
<td>Exposing SFV to u.v. light for 1 h</td>
<td>2</td>
<td>134</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>230</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>254</td>
<td>118</td>
</tr>
<tr>
<td>Exposing mock-infected cells to cycloheximide (5 μg/ml) for 2 h before sampling</td>
<td>95†</td>
<td>15†</td>
<td></td>
</tr>
</tbody>
</table>

*BHK-21 cells were infected with treated or untreated SFV or mock-infected, and dGlc uptake measured at various times after infection as described in the legend to Fig. 1. Protein synthesis was measured by exposing cells to [35S]methionine and measuring TCA-insoluble 35S after various times of incubation. All values quoted are means derived from three separate measurements.

† Treated value as percentage of untreated value.

as the release of virus or cytopathic effects (data not shown). Heat treatment did not entirely abolish the increased dGlc uptake, in that the effect began to be discernible at later times after infection; cytopathic effects were likewise delayed with this heat-treated preparation. The increased uptake of dGlc is not the result of an inhibition of host protein synthesis, because treating uninfected cells with cycloheximide did not alter dGlc uptake (Table 1).

**DISCUSSION**

The present results with VSV- and SFV-infected BHK-21 cells, and similar studies with cells infected with myxoviruses (Pasternak *et al.*, 1982), do not support the hypothesis that "... many if not all cytopathic viruses induce membrane leakiness in their host cell during their life cycle ..." (Carrasco & Smith, 1980), at least not at a time by which virus release is already occurring at maximal rate. However, membrane leakiness was inferred by Carrasco & Smith (1980) from the ability of normally non-permeant compounds to inhibit protein synthesis in infected, but not uninfected, cells. The uptake of one of these compounds, namely the GTP analogue guanylyl (β,γ-methylene)diphosphonate (Carrasco, 1978) by BHK-21 cells has now been measured (M. A. Gray, L. Rodrigues & C. A. Pasternak, unpublished experiments): no difference between SFV-infected and uninfected cells was found; there is, however, a reduced content of GTP in infected cells, as has been noticed by others (Whitehead *et al.*, 1981), and this would account equally well for the observed increase in sensitivity to guanylyl (β,γ-methylene)diphosphonate by infected cells.

The other conclusion to be drawn from our results is that the uptake of glucose is stimulated by infection with VSV and SFV. This raises several questions. What is the biological significance of the change? Is it related, for example, to an increased requirement for preformed sugar in order to synthesize the glycoproteins of the viral envelope (Compans & Klenk, 1979) at maximal rate? Certainly infection by SFV (Kaluza, 1975) or VSV (Turco, 1980) of glucose-starved cells leads to the synthesis of aberrant viral glycoproteins. Is the genetic basis one of derepression of the transport protein caused by an increased rate of protein glycosylation, or is it due to a direct action of a viral gene? Is the mechanism by which it is brought about similar to that by which an increased uptake of glucose is elicited by, for example, insulin (Kono, 1982)? What does appear evident is that the present findings, in concert with other evidence (e.g. Pouyssegur *et al.*, 1980), necessitate a reinterpretation of those hypotheses (see White *et al.*, 1981) that postulate a causal relationship between glucose uptake and cell proliferation.
Membrane alteration in virus infection

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REFERENCES


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