The Use of Osmotic Shock for the Inoculation of Barley Protoplasts with Brome Mosaic Virus

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SUMMARY

The infection of barley protoplasts with brome mosaic virus (BMV) was greatly influenced by osmotic shock produced in protoplasts. Increase of osmotic pressure of the medium immediately before or during inoculation enhanced infection. The efficiency of infection increased with increasing change in mannitol concentration. In contrast, when the osmotic pressure of the medium was decreased before inoculation, few protoplasts were infected even though the other conditions were optimal for infection with BMV. When applied after inoculation, however, decrease of osmotic pressure of the medium had little effect on infection.

In the infection of mammalian cells with ribonucleic acid (RNA) isolated from poliovirus (Koch, 1973), of bacterial protoplasts with RNA from bacteriophage f2 (Engelhardt & Zinder, 1964) and of tobacco protoplasts with RNA from tobacco mosaic virus (TMV; Aoki & Takebe, 1969), osmotic shock produced in the cells plays an important role in infection, and decrease of osmotic pressure of the medium greatly enhances infection. In previous work we found that barley protoplasts isolated in mannitol solution less concentrated than that used for inoculation were more efficiently infected with BMV than when the mannitol concentration was the same throughout or higher than that used for inoculation (Okuno & Furusawa, 1977). In this paper we examine in more detail the influence of osmotic shock produced in barley protoplasts on infection with BMV.

A standard strain of BMV (ATCC 66) was used. Virus growth, purification and storage were as described by Okuno & Furusawa (1978). Protoplasts were isolated from the first leaves of barley (Hordeum vulgare L. cv. Moore) with 1% cellulase (Onozuka R-10; Kinki Yakult Manuf. Co.) in mannitol solution as reported previously (Okuno & Furusawa, 1977). The method used for the inoculation of protoplasts with BMV was basically similar to that of Okuno & Furusawa (1978) except for the procedure used for some samples in the experiment shown in Table 2 (see footnote). Isolated protoplasts were collected by centrifugation (100g, 3 min) and washed twice with mannitol solution by centrifugation (pre-washing). Protoplasts pelleted in 10 ml Wassermann test tubes were suspended in 5 ml of mannitol solution and immediately mixed with an equal volume of mannitol solution of the same concentration containing 40 μg/ml BMV, 0.4 μg/ml poly-L-ornithine (mol. wt. 122000, Sigma Chemical Co.) and 0.2 mM-potassium citrate buffer, pH 5.0. The inoculation mixture was kept at 23 °C for 15 min. Inoculated protoplasts were collected by centrifugation and washed twice with mannitol solution containing 10 mM-CaCl₂ by centrifugation (post-washing). Protoplasts were then incubated in medium according to the method of Okuno & Furusawa (1977). Infection was assessed by fluorescent antibody staining (Okuno et al. 1977; Okuno & Furusawa, 1978).

Barley protoplasts are very stable in mannitol at concentrations above 0.5 M (Okuno & Furusawa, 1977). Increase of osmotic pressure of the medium did not affect the stability of protoplasts at all. A small proportion of protoplasts, however, were sometimes
Table 1. The influence of osmotic shock on the infection of barley protoplasts with BMV

<table>
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<th>Expt.</th>
<th>mannitol molarity during</th>
<th>Time after inoculation (h)</th>
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<td>43</td>
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* Figures are % fluorescing protoplasts determined by fluorescent antibody staining.

aggregated and broken by decreasing osmotic pressure of the medium especially in the presence of BMV (20 to 40 μg/ml). The rest of the aggregated protoplasts were as stable as those of the other samples. In the present experiments 75 to 85% and 60 to 75% of protoplasts were intact after 1 day and 2 days of incubation, respectively.

The infection of barley protoplasts with BMV was greatly influenced by osmotic shock. Infection was enhanced by increasing the osmotic pressure of the medium at the beginning of inoculation (Table 1, Expt. 1). The efficiency of infection increased with increase of concentration of mannitol in the inoculum from 0.5 to 0.85 M using protoplasts isolated and pre-washed with 0.5 M-mannitol (Table 1, Expt. 2). In contrast, infection was almost completely inhibited by decreasing the osmotic pressure of the medium either at pre-washing or at the beginning of inoculation. The influence of the decrease of osmotic pressure of the ice medium at pre-washing was largely reversed by increasing the osmotic pressure at the beginning of inoculation (Table 1, Expt. 1). Infection was less influenced by osmotic shock at post-washing. Increase of osmotic pressure enhanced infection but to a small degree, while decrease of mannitol concentration from 0.7 to 0.5 M often slightly increased the efficiency of infection (Table 1, Expt. 3 and 4). The slight increase may be caused by the osmotic shock when transferring protoplasts post-washed with 0.5 M-mannitol into incubation medium containing 0.7 M-mannitol. The lack of inhibition of infection by the
Table 2. Effect of the timing of increase in mannitol concentration on infection of barley protoplasts with BMV*

<table>
<thead>
<tr>
<th>Treatment no.</th>
<th>Relative time (min)</th>
<th>% fluorescing protoplasts†</th>
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<td>9</td>
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<td>39</td>
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* Protoplasts were inoculated as described in the text and the concentration of mannitol was increased at pre-washing (Treatment no. 1), immediately before inoculation (no. 2), at post-washing (no. 8) or was not increased (no. 9). Protoplasts were suspended directly in 10 ml of 0.7 M-mannitol containing 20 µg/ml BMV, 0.2 µg/ml poly-L-ornithine and 0.1 mM-potassium citrate buffer, pH 5.0 (no. 3). To 6 ml of inoculation mixture was added 4 ml of 1 M-mannitol containing 20 µg/ml BMV, 0.2 µg/ml poly-L-ornithine and 0.1 mM-potassium citrate buffer, pH 5.0, at the stated times (no. 4, 5, 6 and 7).
† Protoplasts were incubated in medium containing 0.7 M-mannitol and infection was determined by fluorescent antibody staining 28 h after inoculation.

decrease of osmotic pressure of medium at post-washing indicates, however, that infection was largely determined before post-washing.

To investigate the effect on infection of the timing of the increase of osmotic pressure of the medium, the concentration of mannitol was increased from 0.5 to 0.7 M at different times before or during inoculation, or at post-washing. Table 2 shows that osmotic shock produced either immediately before or during inoculation was equally effective whereas the effect was small when the osmotic pressure of the medium was increased at pre-washing or post-washing.

Engelhardt & Zinder (1964) reported that the infection of bacterial protoplasts with bacteriophage RNA was greatly enhanced by the osmotic shock produced by adding RNA in water. A similar result was reported by Aoki & Takebe (1969) in the TMV RNA-tobacco protoplast system. In the poliovirus RNA-HeLa cell system, Koch (1963) reported that cell-bound RNA was converted from a ribonuclease (RNase)-sensitive to a RNase-resistant state by transferring the cells from hypertonic to isotonic medium and concluded that RNA entered the cells during the osmotic shock together with the passive influx of ambient solution. As demonstrated in the present study, however, the influence of osmotic shock on the infection of barley protoplasts with BMV is quite opposite to that on infection with virus RNA in these other systems. These findings and our recent electron microscopic observations indicating that probably an endocytotic process is responsible for infection of barley protoplasts with BMV, suggest that the infection of barley protoplasts with BMV did not occur as the result of a simple physical process such as entry of RNA into cells. It is conceivable that the increase of osmotic pressure of the medium causes a shrinkage of protoplasts which may make the membrane structure and properties more favourable for adsorption and uptake of virus particles into protoplasts by an endocytotic process. Alternatively, some physiological change in protoplasts produced by the osmotic shock may favour the initiation of infection. Premecz et al. (1977) reported that RNase activity in isolated tobacco protoplasts was enhanced by incubating protoplasts in hypertonic medium and concluded that the osmotic shock had triggered de novo synthesis of the
enzyme by affecting the physiological state of the cells. This may partly explain the inhibition of infection with RNA. However, the results shown in Table 2 indicate that such a physiological change in cells was not likely to be involved in the infection of barley protoplasts with BMV. Further work will be needed to elucidate the difference in the role of osmotic shock in infection with virus or with virus RNA.

A maximum of about 70% of barley protoplasts became infected with BMV in the experiments described in this paper; this figure is smaller than those for some other virus-protoplast systems (see Takebe, 1975). This may be explained by heterogeneity of protoplasts obtained by the one-step procedure of incubation with cellulase of leaves lacking their abaxial epidermis and/or by postulating that mesophyll cells of monocotyledonous plants are more heterogeneous in metabolic state than those of dicotyledonous plants. However, the method described in this paper may prove to be of use in other virus-protoplast systems and may provide a useful clue in studies on the mechanism of infection of protoplasts with virus.

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REFERENCES


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