Comparative Growth and Selection of Small Plaque and Large Plaque Encephalomyocarditis Virus in the Absence of Inhibitors from Agar

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(Accepted 17 September 1966)

SUMMARY

During multicycle growth of clones of encephalomyocarditis (EMC) virus forming large plaques (EMC/r), small-plaque-forming virus (EMC/r+) appeared and was always selected by conditions in the growth medium to become the predominant plaque type. EMC/r arose but was never selected by conditions in the growth medium to predominance in virus pools initiated by EMC/r+ virus clones. Comparison of virus growth showed that EMC/r+ was produced and released from EMC/r+ infected L cells earlier and in 3- to 5-fold higher yields than was EMC/r from L cells infected with EMC/r. Thus, when separately infected cells are considered, selection strongly favoured EMC/r+ progeny.

In mixed infection of L cells with EMC/r+ and EMC/r, it was shown that:
1) Selection favouring the EMC/r+ virus was reduced as the exposed multiplicity/cell of EMC/r+ virus increased from 0.01 to 5.0 p.f.u./cell.
2) At 1.0 p.f.u./cell EMC/r and 5.0 p.f.u./cell of EMC/r+ less virus of both types was formed in 8 hr than in controls with each virus alone at the same multiplicity.
3) Eight hr after infection more EMC/r was produced and/or released at 0.01 or 0.1 p.f.u./cell EMC/r and 1.0 or 5.0 p.f.u./cell of EMC/r+, than in control cultures without EMC/r+ virus.

"Pure" (> 99 %) pools of the EMC/r virus strain containing 10^8 to 10^10 p.f.u. were produced consistently by increasing the EMC/r virus pool by growth in consecutive single cycle steps, thus eliminating the selective conditions of multicycle growth.

INTRODUCTION

Large-(r) and small-(r+) plaque-forming variants of encephalomyocarditis (EMC) virus similar to those described by Takemoto & Liebhaber (1961) have been isolated (Breeze, 1964). The type of plaque formed has been shown to depend on the effect on the virus particles of sulphated polysaccharide inhibitors in the agar overlay of the plaque assay. Variants which were affected formed small (r+) plaques, whilst those which were not formed large (r) plaques (Liebhaber & Takemoto, 1963).

This paper deals with the comparative growth and selection of EMC/r+ and EMC/r in the absence of sulphated polysaccharide inhibitors. The mutability of EMC/r+ is the subject of the accompanying paper (Breeze & Subak-Sharpe, 1967).
METHODS

**Virus**

The EMC/r⁺ virus* originated from a small-plaque-forming EMC virus stock obtained from the National Institute for Medical Research, Mill Hill, London, England, who had themselves obtained the strain from Dr F. K. Sanders. After the EMC/r⁺ virus strain had been cloned, the EMC/r virus* strain was isolated as a freshly arisen mutant from the r⁺ strain. The EMC/r virus strain was then also cloned before the experiments recorded below were performed.

**Cells**

L cells were obtained from Professor John Paul (Institute of Biochemistry, University of Glasgow) and were recloned by the technique of Puck & Marcus (1955).

**Media**

The medium normally used for growing and maintaining cell cultures consisted either of 8 parts Eagle's medium, 1 part Difco tryptose phosphate broth and 1 part calf serum (ETC) or of 8 parts Eagle's medium and 1 part calf serum (EC). A gas phase of 5% (v/v) CO₂ in air was used with these two media, giving a pH about 7.2 to 7.4 at equilibrium.

The plaque assay overlay medium was made by adding 25 ml. of melted 3.6% Difco Bacto agar (batch no. 01401-01) to 75 ml. Eagle's medium (×1.3 normal strength) plus 5 ml. of calf serum. A staining overlay containing neutral red was made up the same way with the addition of 3 ml. of 0.4% neutral red solution.

Tris saline contained per litre: NaCl 8.0 g., KCl 0.38 g., Na₂HPO₄ 0.1 g., dextrose 1.0 g., 2-amino-2-hydroxymethylpropane-1,3-diol (tris) 3.0 g., phenol red 0.015 g., penicillin 0.1 g., and streptomycin 0.1 g. It had a pH of 7.4, was sterilized by filtration and stored at 4°C.

Trypsin (Difco) was made up to 0.25% in tris saline. It had a pH of 7.6, was sterilized by filtration and stored at -10°C.

**Plaque assay**

The plaque assay system routinely used was of the classical monolayer type (Dulbecco, 1952). Monolayers of L cells were prepared by adding 3.5 x 10⁶ cells in 4 ml. EC or ETC medium to 50 mm. Petri dishes. The plates were used after 12 to 36 hr incubation at 37°C in a humidified atmosphere containing about 5% CO₂. Virus dilutions were made in EC or ETC medium. Aliquots of 0.1 ml. were placed on the drained monolayers and the virus allowed to adsorb for 1 hr at 37°C in the incubator. Each culture then received 5 ml. of overlay medium. This was allowed to set at room temperature and incubation at 37°C continued for 24 to 30 hr. Two ml. of staining overlay were then added to each Petri dish and allowed to set. After further incubation at 37°C, plaques were counted at 36 hr. When very large numbers of cultures were involved, it was found convenient to fix the cells at this time by adding a few ml. of 10% (v/v) formol saline to each. The agar could then be removed and the plaques counted later without the danger of cell lysis and loss of colour contrast.

* EMC/r⁺ and EMC/r were formerly designated s and m respectively (Breeze, 1964).
**Virus cloning technique**

Monolayers infected with limit dilutions of virus were overlayed and incubated for 3 days, when a staining overlay was added. After a further 24 hr incubation cultures were selected which contained only a single plaque. The plug of agar immediately over such a single plaque and the cells forming the plaque itself were transferred with a sterile Pasteur pipette into 1 ml. ETC medium in a small screw-capped vial. Each plaque was separately treated and stored at \(-70^\circ\). Each plaque-isolate was subsequently diluted and inoculated to fresh monolayers at limit dilution so that single plaques were again obtained. The whole process was then repeated for a third time with these plaques. The virus obtained in the third successive single plaque isolate was considered to be purified and to be derived from one infectious virus genome, i.e. to be a clone.

**Infective centre assay**

The medium was removed from two monolayer cultures, one inoculated with each plaque type, and each cell sheet washed twice with 1 ml. cold tris saline. Trypsin (2 ml.) was added to each culture at room temperature. When the cells were individually rounded the trypsin was carefully removed. The cells were then pipetted to produce a monodisperse suspension in EC medium and counted. Suitable dilutions of the cells in one ml. aliquots of agar overlay held at 44° were seeded on to L cell monolayers. When this agar had set a further 4 ml. of overlay medium was added. Subsequent conditions of incubation and staining were those of the plaque assay.

**Growth studies**

**Single cycle experiments.** Monolayer cultures of \(3.5 \times 10^6\) L cells in 50 mm. Petri dishes were exposed to 5 p.f.u./cell of either EMC/r\(^+\) or EMC/r in a 0·1 ml. inoculum (time = 0 hr). The virus was allowed to adsorb for 1 hr at 37°. The inocula were then removed and the monolayers washed 5 times with warm EC medium. Four ml. of warm EC medium were then added to each plate and the incubation continued at 37° in a humidified CO\(_2\) incubator. At various times after infection 2 cultures infected with EMC/r and 2 with EMC/r\(^+\) were removed from the incubator and chilled. The supernatant medium was carefully removed and stored at \(-70^\circ\). (Even 11 hr after infection < 5 % of the cells were contaminating the medium fraction.) The cells were taken off the Petri dishes with 1 ml. trypsin. The Petri dishes were washed with a further 1 ml. of trypsin and the two fluids pooled. The trypsin + cell mixtures were frozen and thawed 3 times, heated to 37° for 1 hr and then stored at \(-70^\circ\). Samples were titrated by standard plaque assay.

**Mixed infection experiment.** This was designed as a ‘checkerboard’ experiment. Monolayers of L cells were simultaneously exposed to a 0·1 ml. inoculum containing either 5·0, 1·0, 0·1, 0·01 or 0 p.f.u./cell of EMC/r\(^+\) and either 1·0, 0·1, 0·01 or 0 p.f.u./cell of EMC/r. The mixtures of EMC/r\(^+\) and EMC/r virus were made immediately before the experiment. After the cultures (in duplicate) were inoculated, they were placed in a humidified CO\(_2\) incubator (time = 0 hr) for 1 hr at 37°. The inocula were then removed and the monolayers washed twice with warm EC medium, then 4 ml./culture
of warm EC medium was added (this took 32 min.), and the cultures returned for incubation at 37°.

After 6 hr incubation, one culture of each pair was removed from the incubator (total time = 8 hr). The medium, and any detached cells it contained, was stored at -70°. The cells were similarly stored, after being detached from the glass in 2 x 1 ml. amounts of trypsin. The duplicate cultures were treated in an identical manner 24 hr after infection. Trypsin + cell mixtures were thawed and frozen twice, and also heated to 37° for 1 hr before virus assay.

Fig. 1. The single cycle growth of EMC/r+ in L cells. Monolayer cultures of 3.4 x 10^6 L cells were exposed to 5 p.f.u. cell in a 0.1 ml. inoculum. Time = zero hours. Adsorption was for 1 hr at 37°. The monolayers were then washed, medium added and incubation continued at 37°. An infective centre assay was performed and replicate cultures were taken at intervals after infection for virus titration. (For further details see text.) ©—©, Total r+ virus; •—•, r+ virus in medium.

Fig. 2. The single cycle growth of EMC/r in L cells. Monolayer cultures of 3.5 x 10^6 L cells were exposed to 5 p.f.u./cell in a 0.1 ml. inoculum. Time = zero hours. Adsorption was for 1 hr at 37°. The monolayers were then washed, medium added and incubation continued at 37°. An infective centre assay was performed and replicate plates were taken at intervals after infection for virus titration. (For further details see text). N.B. Relative displacement in scale on p.f.u. Infective Centre axis compared with Fig. 1. △—△, Total r virus; ▲—▲, r virus in medium.

Fig. 3. Comparison of the rates of virus production between EMC/r+ and EMC/r. Data taken from Figs. 1 and 2. The total virus per culture of EMC/r+ is plotted as a percentage of the 11 hr yield of EMC/r+. The points for EMC/r are similarly plotted with respect to the 11 hr yield of EMC/r. ◦, EMC/r+; △, EMC/r.

RESULTS

Preliminary experiments using BHK21/C13 cells, BHK21/C13/Py6 cells (Stoker & Macpherson, 1964), and L cells showed that neither large-nor small-plaque EMC virus bred true whether or not the virus had been critically cloned. Small-plaque-forming virus appeared during the growth of cloned and uncloned populations of large-plaque-forming virus and soon (always within 3 or 4 passages) became the pre-
dominant plaque type. In populations of small-plaque-forming virus, large plaque virus was generally present at levels between 1 in 3500 and 1 in 4500 p.f.u., but large-plaque-forming virus never became the predominant plaque type on further passaging. It always occurred at this same ‘background’ level.

Single cycle growth curve experiments for EMC/r+ and EMC/r were made simultaneously as described in Methods; 66.7% of cells infected with EMC/r and 35.4% of cells infected with EMC/r+ gave rise to infectious centres 36 hr after inoculation. The yield per infective centre was 10-fold higher for EMC/r+ than for EMC/r (Figs. 1, 2). (The yield per culture was actually fivefold higher, but there was also the twofold difference in infective centres.) The rates of production of EMC/r+ and EMC/r viruses were apparently identical, but the production of EMCr+ occurred up to 30 min. earlier than that of EMC/r (Fig. 3).

Earlier production is not in itself a selective advantage unless the virus produced infects more cells sooner. To achieve this the rates of virus release and the interval between virus production and release must also at least be similar. The horizontal distances between the lines of virus production and release into the medium (Figs. 1, 2) show that EMC/r+ was released earlier than EMC/r. The ratio of the concentration of EMC/r+ in the medium to the concentration of EMC/r in the medium showed approximate equality or slight excess of EMC/r+ during the first 6 hr. From 6½ to 8½ hr, i.e. for a period of 2 hr, a 30- to 200-fold excess of EMC/r+ over EMC/r in the medium was available for fresh cell infection. Then, when nearly all EMC/r+ had been released, the continuing release of EMC/r into the medium resulted in a fall in the ratio. When EMC/r release also ceased, the ratio finally decreased, reflecting the higher final yields (usually 3- to 5-fold) of small-plaque-forming virus in L cells (Fig. 4). (Extraction of debris from virus infected cells with various enzymes, detergents and buffer solutions of several pH values showed that the yield difference was not the result of different amounts of each virus remaining associated with debris (Breeze, 1964)). If this situation also applies generally in cultures where EMC/r+ and large-plaque-forming mutants of it are present together, then EMC/r+ has a very large
selective advantage. It should apply wherever EMC/r+ and EMC/r infect separate cells, but cultures where both virus strains infect the same cell and multiply in it may behave differently.

This situation was then tested by a mixed infection experiment as described in Methods (Tables 1, 2). The 8 hr and 24 hr control results confirmed the previous findings. The 24 hr data from mixed infection are fully explicable in terms of selection in multicyle growth. Three main points arising from this experiment are considered here in some detail.

Table 1. *The results* of the mixed infection experiment 8 hr after infection with EMC/r+ and EMC/r

<table>
<thead>
<tr>
<th>Infection</th>
<th>EMC/r+</th>
<th>EMC/r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Selection Factor</td>
<td>$\frac{\text{Total p.f.u. EMC/r+ \times Exposed multiplicity to EMC/r}}{\text{Total p.f.u. EMC/r \times Exposed multiplicity to EMC/r+}}$</td>
<td></td>
</tr>
<tr>
<td>$0.1r^+$</td>
<td>$0.01r+$</td>
<td>$0.0r$</td>
</tr>
<tr>
<td>$5.0r^+$</td>
<td>$4.0r$</td>
<td>$3.0r$</td>
</tr>
<tr>
<td>$1.0r^+$</td>
<td>$0.1r$</td>
<td>$0.01r$</td>
</tr>
</tbody>
</table>

* Expressed as the number of p.f.u. of EMC/r+ and of EMC/r in cells (c) and supernatant (s) per culture.

† Infection of the monolayer cultures was for 1 hr at 37°C at the exposed multiplicities of r+ and r indicated at the sides and heads of columns respectively. The monolayers were then washed and incubated with EC medium at 37°C. Eight hr after infection the medium and cells were separated, the cells harvested with trypsin for subsequent virus assay (for further details see text).

The first concerns the problem of selection. For convenience a term ‘Overall Selection Factor’ is here defined as

$$\frac{\text{Total p.f.u. EMC/r+}}{\text{Total p.f.u. EMC/r \times Exposed multiplicity to EMC/r}} \times \frac{\text{Exposed multiplicity to EMC/r}}{\text{Exposed multiplicity to EMC/r+}}$$

(if > 1 this indicates selective advantage of EMC/r+). The observed value O for this factor can be calculated for each plate receiving a particular mixture. In addition the expected value of the overall selection factor, E, can be calculated using the total titre of the control receiving the same quantity of EMC/r+ but no EMC/r, and the control receiving the same quantity of EMC/r but no EMC/r+. This E value is the expectation assuming that EMC/r and EMC/r+ do not interact under conditions of
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joint infection. The observed values indicate that EMC/r+ is virtually always at a selective advantage, but that the amount of selection against any particular multiplicity of EMC/r decreases in mixed infection as the exposed multiplicity of EMC/r+ increases. The expected values are in accord with this trend (Table 3).

Table 2. The results* of the mixed infection experiment 24 hr after infection with EMC/r+ and EMC/r

<table>
<thead>
<tr>
<th>EMC/r+ multiplicity</th>
<th>1:0 r</th>
<th>0:1 r</th>
<th>0:01 r</th>
<th>0:0 r</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:0r+</td>
<td>2:03 × 10^9 r+</td>
<td>3:30 × 10^9 r+</td>
<td>3:42 × 10^9 r+</td>
<td>1:57 × 10^9 r+</td>
</tr>
<tr>
<td></td>
<td>(or in 47r+)</td>
<td>(or in 47r+)</td>
<td>(or in 114r+)</td>
<td></td>
</tr>
<tr>
<td>1:0r+</td>
<td>4:14 × 10^9 r+</td>
<td>7:25 × 10^9 r+</td>
<td>6:94 × 10^9 r+</td>
<td>5:70 × 10^9 r+</td>
</tr>
<tr>
<td></td>
<td>(or in 90r+)</td>
<td>(or in 90r+)</td>
<td>(or in 47r+)</td>
<td></td>
</tr>
<tr>
<td>0:1r+</td>
<td>4:40 × 10^9 r+</td>
<td>4:86 × 10^9 r+</td>
<td>8:00 × 10^9 r+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(or in 354r+)</td>
<td>(or in 354r+)</td>
<td>(or in 354r+)</td>
<td></td>
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</table>

Table 3. Observed and expected values for the overall selection factor* 8 hr after infection

<table>
<thead>
<tr>
<th>EMC/r+ multiplicity</th>
<th>1:0 r</th>
<th>0:1 r</th>
<th>0:01 r</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:0r+</td>
<td>2:8</td>
<td>2:0</td>
<td>0:9</td>
</tr>
<tr>
<td></td>
<td>(or in 12r)</td>
<td>(or in 31r)</td>
<td>(10r+ in 95r)</td>
</tr>
<tr>
<td>1:0r+</td>
<td>1:2</td>
<td>1:2</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>(or in 200r)</td>
<td>(or in 200r)</td>
<td>(2r+ in 200r)</td>
</tr>
<tr>
<td>0:1r+</td>
<td>3:0</td>
<td>3:1</td>
<td>1:2</td>
</tr>
<tr>
<td></td>
<td>(or in 113r)</td>
<td>(or in 113r)</td>
<td>(3r+ in 113r)</td>
</tr>
<tr>
<td>0:0r+</td>
<td>6:2</td>
<td>7:7</td>
<td>7:1</td>
</tr>
<tr>
<td></td>
<td>12:7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0:01r+</td>
<td>9:0</td>
<td>8:6</td>
<td>15:5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Overall Selection Factor = \( \frac{\text{total p.f.u. EMC/r}^+}{\text{total p.f.u. EMC/r}} \times \frac{\text{exposed multiplicity EMC/r}}{\text{exposed multiplicity EMC/r}^+} \).

† Observed values; computed from r+ and r titres for each mixedly infected culture given in Table 1.

‡ Expected values; computed from r+ and r titres given in Table 1 of cultures which received EMC/r+ alone and EMC/r alone at the same multiplicities of r+ and r as did the corresponding mixedly infected culture.
The possibility of interaction following joint infection can be estimated by comparison of observed and expected values. If the differences between paired 'observed' (O) and 'expected' (E) values are not considered significant, then the changing selection against EMC/r with multiplicity of exposure to EMC/r+ is fully explained as due to the relative efficiencies of p.f.u. production of EMC/r+ and EMC/r alone at the different multiplicities used. If paired value differences are significant, this implies interaction following mixed infection. Unfortunately, no completely satisfactory method has been found to calculate the standard errors of these values.

Fig. 5. The effects of mixed infection on the amount of EMC/r virus formed 8 hr after infection. The data, taken from Table 1, plotted as the ratio of the amount of EMC/r virus formed in mixed infection to the amount of EMC/r virus formed in the control culture (infected at the same multiplicity with EMC/r alone), against the exposed multiplicity of EMC/r+ virus in the mixedly infected culture. ○, 1.0 p.f.u./cell; •, 0.1 p.f.u./cell; △, 0.01 p.f.u./cell. The points referred to the same control are joined together by lines as shown.

Fig. 6. The effects of mixed infection on the amount of EMC/r virus released 8 hr after infection. The data, taken from Table 1, are plotted as the ratio of the amount of EMC/r virus released in mixed infection to the amount of EMC/r virus released in the control culture (infected at the same multiplicity with EMC/r alone), against the exposed multiplicity of EMC/r+ virus in the mixedly infected culture. The symbols are used as in Fig. 5.

The second point concerns the effect of double infection on the yield of EMC/r. At the lowest multiplicity of EMC/r per cell, when the exposed multiplicity per cell of EMC/r+ was either 5.0 or 1.0, there was a highly significant rise, as a direct result of the mixed infection, in both the amount of EMC/r virus formed (Fig. 5), and even
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more so in the amount released into the medium (Fig. 6). The greater quantity and
earlier release of EMC/r here (and also release of EMC/r when its initial multiplicity
was 0·1 p.f.u./cell) could mean that EMC/r+ RNA predominated in the host cells,
resulting in the control of the virus maturation processes by proteins specified by
EMC/r+. Thus the possibility that some of the EMC/r virus RNA produced may be
wrapped in an EMC/r+ protein coat, or in a mixed EMC/r+-EMC/r protein coat
(Ledinko & Hirst, 1961) must be borne in mind. This could be critically investigated
by electrophoresis (Thorne, 1963; Breeze, 1964; Breeze & Thorne, 1966). It is not
understood why mixed infection with an EMC/r multiplicity of 1·0 p.f.u./cell gives
the opposite result, particularly when EMC/r+ multiplicity is 5·0 p.f.u./cell. Generally
the final total 24 hr titre that was reached when EMC/r+ predominated was about
10^9 p.f.u. and when EMC/r predominated it was about 5×10^8 p.f.u.

The third point concerns the interaction of EMC/r and EMC/r+ growing in the
same cell for one growth cycle (Table 2). At the highest multiplicities used, 1·0 p.f.u./
cell EMC/r and 5·0 p.f.u./cell EMC/r+, and at 0·1 p.f.u./cell EMC/r and 5·0 p.f.u./cell
EMC/r+, significantly less virus of both types was formed than expected. There was
no evidence of interference at lower multiplicities of EMC/r+ and EMC/r.

All the results indicated that EMC/r clones failed to breed true because of a high
r → r+ mutation rate (see Table 1, EMC/r controls) combined with rapid selection
favouring EMC/r+ (see Table 2, EMC/r controls). Nothing can be done about a high
mutation rate, but the selective conditions of multicycle growth can be eliminated.
When this was done by multiplying up the main virus pool in four separate successive
single growth cycle steps, the predominance of EMC/r was maintained. At the end
of each single cycle, the virus was released from the cells and all of it made available
for a fresh cycle of cell infection by exposing the cells to virus at 37° for 1 hr at an
average multiplicity of 1 p.f.u./cell. Eight hr later the whole cell culture was frozen
and thawed and the clarified supernatant fluid used as the virus pool for the next
cycle. Four main EMC/r pools were prepared in this manner. In every case the final
virus pool contained between 10^9 and 10^{10} p.f.u. and in no case were r+ plaques
observed, that is, they contained < 1·0 % EMC/r+.

DISCUSSION

Higher titres of small-plaque-forming virus were obtained in all cell lines used
compared with the titres of large-plaque-forming virus. Unfortunately, no information
was obtained by electron microscopy about particle: infectivity ratios of the large- and
small-plaque-forming types. Attempts made to determine these ratios were frustrated
by comparatively low yields and impure concentrated preparations, even after chroma-
tographic and enzymic purification (Breeze, 1964).

Adsorption rates of these strains were not compared since Takemoto & Liebhaber
(1961) reported that they found no difference in the rates of adsorption of large- and
small-plaque-forming EMC virus to L cells. Thus any adsorption rate advantage
of EMC/r+ could only be slight, and could exert at most a very minor effect on the
rapid selection of small-plaque types in large-plaque-forming virus stocks.

EMC/r+ was produced some minutes earlier than EMC/r, although at the same
rate (Fig. 4). This earlier production of EMC/r+ compared with EMC/r could be
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a reflexion of more rapid effective penetration by EMC/r⁺, rather than a reflexion of later differential intracellular events. Thus adsorption and penetration problems warrant re-examination. EMC/r⁺ was released far sooner than EMC/r when the single cycle growth of the two types was studied. This probably also applies in mixed infection at low multiplicity where almost all cells are singly infected with one plaque type only. Thus earlier release and higher yields of EMC/r⁺ per cell are considered the two principal reasons why large plaque mutants of EMC virus are selected against so that they occur at relatively low levels (c. 1 in 2000 to 5000) in pools of small-plaque-forming virus.

The author gratefully acknowledges the patient instruction, constructive suggestions and encouragement given him by Dr H. Subak-Sharpe. He is also indebted to Professor Michael Stoker, Dr Ian Macpherson and other friends and colleagues in Glasgow, Scotland.

This work was performed during the tenure (1961 to 1964) of a Medical Research Council Scholarship for Training in Research Methods.

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(Received 30 August 1966)