Further Studies on a Purification Procedure for Encephalomyocarditis Virus

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The procedure used in this laboratory for the purification of encephalomyocarditis (EMC) virus has been described by Burness (1969a). Contamination with host cell material was shown to be absent by the lack of radioactivity in purified virus when homogenates of uninfected host cells containing protein and nucleic acid labelled with [3H]amino acids and 32P, respectively, were added to the crude virus before purification (Burness, 1969a). Although several methods were employed to check the purity of the product, contamination with proteins unique to infected cells, such as non-structural virus proteins was not excluded. We describe here a procedure to investigate and virtually exclude such possible contamination, by using EMC virus variants separable from one another by calcium phosphate chromatography.

In the original description of the procedure it was suggested that ribosomes would be the most likely subcellular contaminants of picornavirus preparations since both kinds of particles have similar biophysical characteristics (Burness, 1969a). However, ribosomes were never detected by ultracentrifugal analysis at any stage of the purification procedure and in this report we show that this is because ribosomes are unstable in the pyrophosphate-containing buffers used during purification.

A pool of a large-plaque-producing variant of EMC virus, known to be eluted from calcium phosphate columns by 0.17 M-phosphate buffer, pH 7.0 (Burness, 1967, 1969b), was grown in Krebs ascites tumour cells as previously described (Sanders, Huppert & Hoskins, 1958; Burness, 1969a) in Earle’s saline containing per litre the following 3H-labelled amino acids purchased from Amersham/Searle Co.: 40 μC each of L-arginine-T(G), HCl, L-3-phenylalanine (ring-4-T) and L-serine-3-T; 80 μC of L-leucine-4,5-T and 100 μC each of L-aspartic acid-T(G) and DL-lysine-4,5-T(N), HCl. A pool of a small-plaque-producing variant of EMC virus, known to be eluted from calcium phosphate columns by 0.43 M-phosphate buffer, pH 7 (Burness, 1967, 1969b), was grown under identical conditions but omitting the radioactive amino acids. When virus growth was complete, both virus pools were combined and the whole subjected to purification (Burness, 1969a), including chromatography on calcium phosphate using a gradient known to separate large-plaque and small-plaque variants (Fig. 1).

If non-structural proteins survived the purification procedure and represented a contaminant adsorbed to purified virus, then the small-plaque variant was as likely to be contaminated with the radioactive, non-structural proteins induced by the large-plaque variant as was the purified large-plaque variant itself. If this were so, both virus peaks eluted from calcium phosphate by 0.17 M- and 0.43 M-phosphate buffer and containing large-plaque and small-plaque variants, respectively, would contain radioactivity. If, however, the non-structural proteins were effectively removed by the purification procedure only the large-plaque variants eluted by 0.17 M-phosphate buffer would contain radioactivity presumably in the virus itself.

Haemagglutinating activity and extinction at 260 nm. due to large-plaque virus and radioactivity were eluted coincidentally by 0.17 M-phosphate buffer, but the peak of
haemagglutinating activity and extinction at 260 nm. due to small-plaque virus eluted by 0.43 m-phosphate buffer contained background levels only of radioactivity (Fig. 1), thus demonstrating no contamination of purified virus with non-structural proteins.

The virus in the two peaks eluted from calcium phosphate was collected separately by ultracentrifugation at 65,000 rev./min. for 30 min. in a Spinco rotor type 65, the virus resuspended in 0.1 M-NaCl + 0.02 M-phosphate buffer, pH 8.0, and the extinction at 260 nm. and radioactivity measured. The specific activity of the large-plaque virus protein was such that 1 count/min. represented 0.017 μg. protein (Table I). Therefore, the radioactivity present in the small-plaque virus peak represented 0.017 μg. protein in a total of 1.5 μg., or about 1% contamination. Most of the radioactivity in the small-plaque virus peak was probably due to cross-contamination with large-plaque virus rather than with non-structural proteins, which therefore would contaminate the small-plaque virus by far less than 1%.

A cytoplasmic extract, prepared from Krebs ascites cells by a lysis procedure (Pogo & Fairferman, personal communication) and suspended in a solution containing 0.25 M-sucrose

Table 1. Radioactivity in small-plaque and large-plaque variant proteins after calcium phosphate chromatography of virus

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Large-plaque variant</th>
<th>Small-plaque variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counts/min.</td>
<td>1610</td>
<td>16</td>
</tr>
<tr>
<td>$E_{260}$</td>
<td>0.310</td>
<td>0.270</td>
</tr>
<tr>
<td>μg. protein*</td>
<td>27.0</td>
<td>24.0</td>
</tr>
<tr>
<td>μg. protein/counts/min.</td>
<td>0.017</td>
<td>1.500</td>
</tr>
</tbody>
</table>

* Calculated from the $E_{260}$ for virus with an $E_{1%}$ at 260 nm. = 77.4 and containing 68.3% protein (Burness, 1970).
+0·1 M-KCl+0·005 M-MgCl₂+0·01 M-β-mercaptoethanol and buffered with 0·01 M-tris, pH 7·6, was subjected to the virus purification procedure. Samples were removed at each step for extinction measurements at 260 nm. and for ultracentrifugal analysis in a Spinco Model E ultracentrifuge.

Most of the material in the original cytoplasmic preparation moved with a sedimentation coefficient, $S_{20,w} = 78$ s, expected for concentrated ribosomes, but there were small amounts of ribosomal subunits with $S_{20,w} = 43$ s and 59 s, respectively, and dimers of $S_{20,w} = 114$ s (Fig. 2a). Acid precipitation followed by resuspension in PP-NaCl (0·1 M-potassium phosphate buffer, pH 8·0 + 0·2 M-sodium pyrophosphate, pH 8·0 + 0·1 M-NaCl) gave 50% recovery of extinction at 260 nm. but complete destruction of the components in the original preparation and the appearance of a trace of a new component with $S_{20,w} = 28$ s (Fig. 2b).

The succeeding purification step involved extraction with organic solvent which reduced the extinction at 260 nm. to 1·8% of the original but did not reduce significantly the amount of the 28 s component as determined by ultracentrifugal analysis. The organic solvent extraction step would normally be followed by ultracentrifugation at 65,000 rev./min. for 30 min. in a Spinco rotor 65, during which the 28 s component would remain in the supernatant fluid, unlike the virus which would sediment. However, this step was omitted in order to leave 28 s material to test its sensitivity to enzyme. The 28 s component was completely degraded by pancreatic RNase (10 μg./ml. at 37° for 30 min.) used in the purification procedure but not by trypsin (250 μg./ml. at 37° for 30 min.), suggesting that the component contained RNA.

The enzyme step is usually followed by a second ultracentrifugation at 65,000 rev./min. for 30 min. in a Spinco rotor 65, which would effectively remove the trace of degraded 28 s component surviving to this stage of the procedure.
Short communications

It was of interest to determine whether acid precipitation, resuspension in PP-NaCl or a combination of both caused the destruction of ribosome integrity to give the 28 s component. Addition of 1 vol. of PP-NaCl to 1 vol. of ribosomes produced a preparation which when examined by ultracentrifugal analysis (Fig. 2c) appeared to be identical with that produced by combined acid precipitation and resuspension (Fig. 2b), thus suggesting that it was the resuspension buffer which caused degradation of ribosomes, presumably owing to the affinity of pyrophosphate in the buffer for divalent cations, the latter being necessary to maintain the integrity of ribosomes. Since this buffer is used throughout the purification procedure, it would explain why ribosomes are never seen as contaminants at any stage of virus purification.

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REFERENCES


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