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USE OF HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY FOR THE SEPARATION OF POLIOVIRUS AND SUBVIRAL PARTICLES

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SUMMARY

The size-dependent separation of viral and subviral particles in the range 10^5–10^7 daltons was undertaken by high-performance liquid chromatography. A combination of Ultrahydrogel 2000 and 1000 size-exclusion columns, equilibrated and developed with Tris buffer (pH 7.4), was used to fractionate extracts of cells infected with radiolabelled poliovirus. Poliovirions (30 nm) and subviral particles (20 nm) were separated according to size with full retention of their biological activities. Procapsids (same size as virions, but devoid of RNA) could not be separated from virions. Sample recoveries as determined with radiolabelled material constantly exceeded 70%. The method was successfully applied to the separation of viral and subviral particles from complex mixtures.

INTRODUCTION

Previous work with soft size-exclusion columns (Sepharose-type support materials) provided a relatively high resolving capacity for macromolecules, but typically required long elution times, as the gel matrix did not withstand the high pressures required for increased flow-rates. Moreover, relatively large amounts of material were required. The resolution of proteins according to their molecular mass has made rapid advances after the introduction of rigid, small-diameter silica-based gels, covalently bonded with hydrophilic compounds. Owing to their improved efficiency (narrower peak widths, higher plate counts), permeability and surface properties, the performance of these gels exceeded that of the soft gels, with typically a 100-fold reduction in separation times[1].

Ultrahydrogel columns (Waters–Millipore, Milford, MA, U.S.A.), which are cross-linked hydroxylated polymers with some residual carboxyl functionality, have been recommended for separating a variety of biological polymers[2]. The molecular mass calibration graphs of these columns are similar to those of the TSK-PW col-

TABLE I

PHYSICAL PARAMETERS OF VIRIONS, PROCAPSIDS AND 14S SUBUNITS

Species of particles	Molecular mass[a]	Sedimentation value[a,b]	Diameter (nm)[c]	Elution volume (ml)[d]
Virions	$8.25 \cdot 10^6$	160S	29	17.4
Procapsids	$5.85 \cdot 10^6$	65S	29	17.4
14S subunits	$4.80 \cdot 10^5$	14S	20	19.6

[a] From ref. 12.
[b] From ref. 13.
[c] From ref. 11.
[d] Using a Ultrahydrogel 2000 + 1000 column combination at a flow-rate of 0.2 ml min^{-1}.

umns manufactured by TosoHaas (Philadelphia, PA, U.S.A.)[2–4], which were successfully applied to the separation of polysaccharides, large enzymes, viruses, ribosomes, DNA, RNA, etc.[5,6] (we chose the Ultrahydrogel material for reasons of economy).

The study of poliovirus morphogenesis requires the separation of viral precursor particles and their assembly products (Table I). Until now, this separation could only be achieved by sucrose gradient ultracentrifugation[7]. We present here an alternative methodology, i.e., the size-dependent separation of virions and subviral particles by size-exclusion high-performance liquid chromatography (HPLC) using a combination of Ultrahydrogel columns.

EXPERIMENTAL

Preparation of radiolabelled extracts of poliovirus-infected cells[8,9]

HeLa cells in suspension (10^7 ml^{-1}) were infected with Mahoney (serotype 1) poliovirus at an input multiplicity of 100 plaque-forming units per cell, and 25 μCi ml^{-1} [^{35}S]methionine were added after 3 h, i.e., after host cell protein synthesis was completely shut-off. The cells were collected 2.5 h later by centrifugation and resuspended to a density of $2 \cdot 10^8$ cells ml^{-1} in RSB buffer (10 mM Tris, 10 mM NaCl, 1.5 mM MgCl$_2$, HCl to pH 7.4). They were freeze–thawed three times and cleared of debris and nuclei by low-speed centrifugation. The supernatant was stored at $-80°$C until use.

Preparation of radiolabelled poliovirons and subviral particles[8,9]

Radiolabelled virions, procapsids and 14S subunits were prepared as described. Briefly, a radiolabelled poliovirus-infected cell extract (as described above) was submitted to ultracentrifugation in a sucrose density gradient (prepared in RSB). The radioactivity profile was determined, and the 160S virions, 65S procapsids and 14S subunits were collected and stored at $-80°$C in 50 000 cpm aliquots. The characteristics of the three species of particles are described in Table I.

High-performance liquid chromatography

High-performance size-exclusion chromatography was carried out at 4°C in an LKB 2203 Minicoldlab refrigerated system (Pharmacia LKB, Bromma, Sweden) on
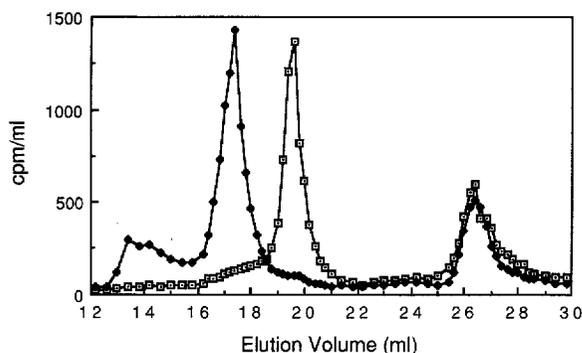
Fig. 1. Elution profile of ^{35}S-labelled 14S viral particles on Ultrahydrogel 2000 + 1000 at 0.2 ml min^{-1} before (□) and after (■) incubation for 20 min at 37°C.

Ultrahydrogel 2000 and Ultrahydrogel 1000 columns (300 × 7.5 mm I.D.) (Waters–Millipore), reported to have polyethylene oxide separation ranges of $5 \cdot 10^4$–$5 \cdot 10^6$ and $5 \cdot 10^3$–$1 \cdot 10^6$ daltons, respectively. Samples were applied with a heparin syringe using a 7000-p.s.i. injection valve (Valco Instruments, Houston, TX, U.S.A.) and a 250-μl loop. The columns were attached to a Waters Model M 45 pump, equilibrated and eluted with RSB of pH 7.4 (filtered with 0.45-μm filter). In all experiments an operating flow-rate of 0.2 ml min^{-1} was used. According to Swergold and Rubin[10], flow-rates exceeding 0.2 ml min^{-1} reduce the efficiency of this type of column. The low back-pressure (40–60 p.s.i.) exhibited by these columns allowed their use in tandem for more efficient separations. The column effluent was monitored at 277 nm with an LKB Uvicord S detector, and collected with an LKB fraction collector. A portion of each eluate fraction was mixed with scintillation liquid to determine the radioactivity profile, using a 1218 Rackbeta liquid scintillation counter and Rackbeta Plot program (LKB).

The total permeation volume (V_t) of the columns was determined from the elution of [35S]methionine (Table II). As no test material of a size sufficient for complete exclusion was available, the total exclusion volume (V_0) was inferred from the sharp rise in absorbance observed when the largest particles were eluted, as illustrated in Fig. 1.

TABLE II

EXCLUDED AND TOTAL VOLUMES OF ULTRAHYDROGEL COLUMNS

Volumes determined at a flow-rate of 0.2 ml min^{-1}.

Column	V_0 (ml)	V_t (ml)
Ultrahydrogel 2000	6.06	13.52
Ultrahydrogel 1000	5.94	12.67
Ultrahydrogel 2000 + 1000	12.00	26.20

RESULTS

Table I gives a survey of the poliovirus-related particles, the separation of which was attempted. Procapsids, which lack RNA, have a much lower (65S) sedimentation coefficient than virions (160S), and can easily be separated by ultracentrifugation. On the other hand, they have the same size (29 nm). When tested in size-exclusion chromatography using Ultrahydrogel 2000 and 1000 columns mounted in series, both species of particles yielded a single main radioactivity peak (Fig. 2) with an elution volume of 17.4 ml. This result confirms that separation was exclusively according to size.

The third species of poliovirus-related particles is the 14S subunit. These particles measure 20 nm in diameter, to a thickness of 6–7 nm[11]. As expected, 14S subunits eluted separately from virions, with an elution volume of 19.6 ml (Fig. 3).

As the chromatographic separation of poliovirus-related particles was intended as an aid for the study of poliovirus assembly, it was important to learn whether the 14S subunits could be separated from the empty capsids, into which they are known to assemble spontaneously at 37°C[7,12]. Therefore, a sample of 14S material was incubated at 37°C for 20 min, with a control sample being kept in the cold. Both samples were then analysed by HPLC. Fig. 1 shows the results; the control 14S subunits eluted at 19.6 ml as previously observed, whereas those which were allowed to assemble into empty capsids eluted at 17.4 ml, the characteristic elution volume of virions and procapsids. Obviously, the size-exclusion HPLC method allows us to follow the progress of the assembly reaction.

An unfractionated extract of poliovirus infected HeLa cells (see Experimental) was chromatographed. The poliovirus infection is known to cause a complete shut-off of host protein synthesis. Therefore, only viral proteins and particles were radiolabelled in the extract. The radioactivity and absorbance profiles are illustrated in Fig. 4. The radioactivity profile showed four peaks, as follows.

(i) The material eluting at 17.4 ml, corresponded to virions and procapsids. This identification was confirmed by ultracentrifugation of the eluate peak fraction (Fig. 5a) and also by antigenicity determination (presence on N1 and N2 epitopes; results not shown).
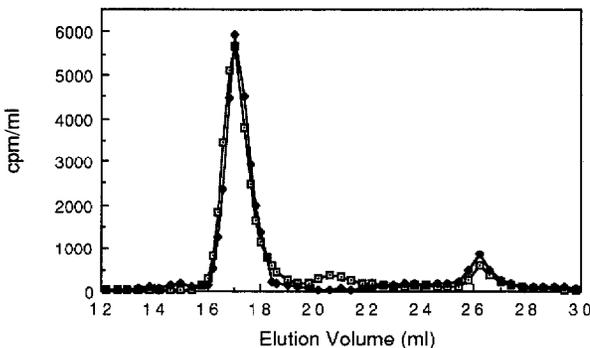

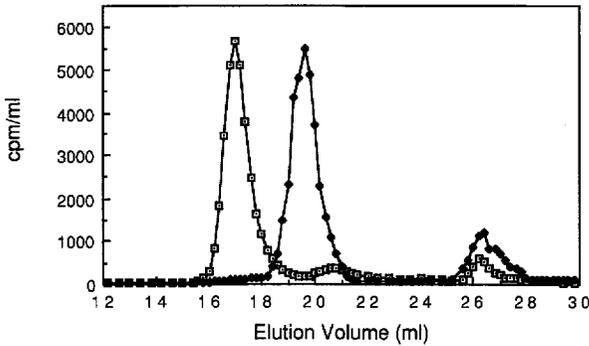Fig. 2. Elution profile of ^{35}S-labelled virions (□) and procapsids (■), eluted as in Fig. 1.

Fig. 3. Elution profile of ^{35}S-labelled virions (□) and 14S subunits (■), eluted as in Fig. 1.

(ii) Similarly, the material eluting at 19.6 ml consisted of 14S subunits (Fig. 5b); as expected, these particles had N1 and H epitopes (not shown).

(iii) The bulk of the radioactivity eluted as an asymmetric peak around 24.5 ml; this elution volume suggested a molecular weight close to 20 kilodaltons. The radioactive material was not precipitable by trichloroacetic acid and presumably consisted of [35S]methionine-loaded tRNA; this tentative identification was confirmed by the similar elution pattern of purified tRNA (Fig. 6).

(iv) The material eluting at 26.2 ml was presumably free [35S]methionine, as this elution volume corresponded to the total volume of the combined columns (300 × 7.5 mm × 2).

The absorbance profile shown in Fig. 4 was remarkable in that some UV-absorbing components eluted past the total volume of 26.2 ml (Table II), indicating that the columns may not function solely in the exclusion mode, but may retain some components by hydrophobic interaction with the gel matrix.

Only a minor decrease in resolution was noted when the volume of the injected mixture was increased from 5 to 100 μl. When the injection volume was further increased to 250 μl, the resolution was significantly decreased as far as the total protein content (shown by the absorbance profile) is concerned. However, the sep-
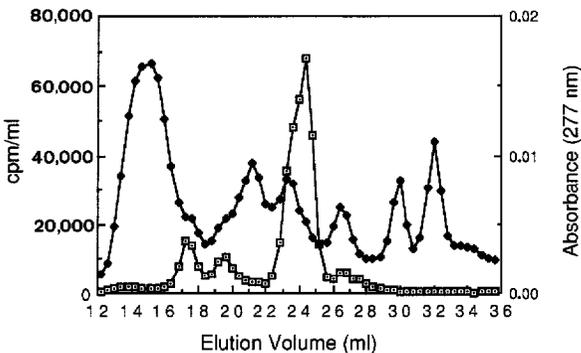

Fig. 4. Elution profile of an extract of infected HeLa cells eluted as in Fig. 1. (□) Radioactive ^{35}S profile in cpm ml^{-1}; (■) absorbance monitored at 277 nm.
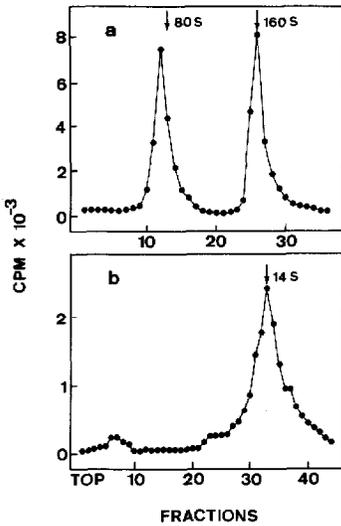
Fig. 5. Sucrose gradient ultracentrifugation of size-exclusion eluate fractions. Two of the fractions shown in the elution profile in Fig. 4 were collected: (a) peak fraction at 17.4 ml and (b) peak fraction at 19.6 ml. Sample a (50 000 cpm) was layered onto a 15–30% sucrose gradient and centrifuged for 5.5 h at 110 000 g_{av} in an MSA 30.6 rotor. Sample b (20 000 cpm) was layered onto a 5–20% gradient and centrifuged for 21 h at 82 000 g_{av} in an MSA 30.3 rotor. Vertical arrows indicate location of 14S subunits, virions (160S) and artificial empty capsids (80S) in control tubes.

aration of the radioactive products (only viral products are radiolabelled, owing to the host shut-off) remained unmodified. At the rate of 0.2 ml min^{-1}, the elution profile of the various viral components was highly reproducible. This was verified in 20 runs, which yielded a precision in retention time of 0.1% (data not shown).

Recovery of input radioactivity, studied using either microgram amounts of [^{35}S]methionine-labelled purified particles or unfractioned cell extracts, always exceeded 70%.

The viability of poliovirus was unaffected by the chromatographic process. In an experiment with ^{35}S-labelled virus, 75% of the input radioactivity and 70% of the
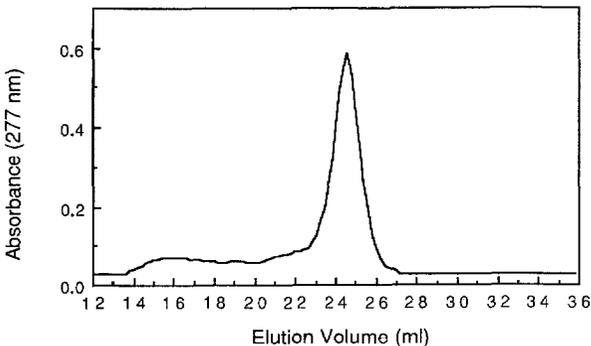

Fig. 6. Elution profile of 100 μg of tRNA (Sigma, St. Louis, MO, U.S.A.) eluted as in Fig. 1.

infectivity (plaque-forming units) were recovered from the virus peak; in other words, the specific infectivities of the recovered and input virus were essentially the same.

DISCUSSION

As demonstrated by Ollivon et al.[6], size-exlusion HPLC allows the quantitation and recovery of viral products with a much better resolution than could be achieved with conventional materials. The present results demonstrate that the combination of Ultrahydrogel 2000 and 1000 columns is suitable for the purification and characterization of viral and subviral particles up to $8 \cdot 10^6$ daltons. The 14S subunit, a crucial intermediate in the morphogenetic pathway of poliovirus, can easily be separated from the larger particles (naturally occuring procapsids, empty capsids and virions).

The process entails less than a 30% loss of input material and does not cause denaturation of virions (retention of infectivity) or 14S subunits (retention of assembly activity). Each run requires approximately 3 h; however, as no material is eluted during the first hour, new injections can be made every 2 h without interference with the previous sample. In comparison, sucrose gradient ultracentrifugation typically requires 5–21 h (Fig. 5), although it allows the simultaneous analysis of up to six samples.

As a preparative tool, size-exclusion HPLC has two major advantages over sucrose gradient ultracentrifugation: the eluate fractions are free of sucrose and even relatively small subviral particles, such as 14S subunits, are completely free of [^{35}S]methionine. In sucrose gradient ultracentrifugation, 14S material is always contaminated by free labelled amino acids, owing to their fast diffusion. The removal of either sucrose or free amino acids then requires a dialysis, which often entails losses of labelled material. In conclusion, size-exclusion HPLC on Ultrahydrogel columns is a useful alternative to sucrose gradient ultracentrifugation for the separation of viral and subviral particles, such as those involved in poliovirus morphogenesis.

ACKNOWLEDGEMENTS

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