Morphological Components of Herpesvirus.  
II. Preservation of Virus During Negative Staining Procedures

By S. K. VERNON*, W. C. LAWRENCE†, G. H. COHEN‡, M. DURSO* AND B. A. RUBIN*

* Department of Biological Product Development, Wyeth Laboratories, Inc. Philadelphia, Pennsylvania 19101 and † Department of Pathobiology, School of Veterinary Medicine, and ‡ Center for Oral Health Research and Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104 U.S.A.

(Accepted 17 December 1975)

SUMMARY

The disruption of envelopes and the fragmentation of capsids of equine herpesvirus type I observed in negatively stained samples were attributed to viral dehydration on carbon films during preparation for electron microscopy. Prior fixation of virus with OsO₄ or glutaraldehyde and subsequent application of negative stain before drying minimized envelope disruption and virtually eliminated the occurrence of capsomere sheets and broken capsids. This simple procedure significantly improves electron microscopic evaluation of herpesvirus samples.

INTRODUCTION

Successful methods of examining herpesviruses by electron microscopy have employed (1) observation of thin sections of fixed, dehydrated virus, (2) observation of viruses contrasted by negative staining (Brenner & Horne, 1959; Wildy, Russell & Horne, 1960), and (3) observation of negatively stained 'pseudoreplicas' of dried virus samples (Sharp et al. 1952).

Because electron microscopy is commonly used to investigate the degradative effects of various treatments on herpesvirus, precautions should be taken to minimize the possibility that alterations in virus structure occur during preparation of samples for observation. That need is demonstrated, in the available literature, by the paucity of electron micrographs showing high proportions of intact negatively stained envelopes or capsid shells.

In our initial study of purified, enveloped equine herpesvirus type I (EHV-1) and herpes simplex virus type I (HSV-I), we observed heterogeneous populations of virus particles in various stages of disruption (Vernon, Lawrence & Cohen, 1974). In contrast, biochemical and infectivity studies suggested that the same samples contained highly purified, stable, infectious virus (Lawrence, 1976). Therefore, we postulated that the degradation of the particles occurred during preparation of the samples for electron microscopy.

This paper describes a simple method to prepare negatively stained herpesviruses for electron microscopy while preserving the structural integrity of the particles.
METHODS

Purified viruses. Enveloped EHV-1 was purified by flotation in CsCl density gradients (Lawrence, 1976). EHV-1 capsids were prepared from detergent-treated infected L cells (W. C. Lawrence, manuscript in preparation). Viruses were stored in CsCl at 4°C for up to 2 weeks without undergoing significant, visible degradation.

Fixation. Virus samples were mixed with equal volumes of 1% (w/v) OsO₄ or 2% (v/v) glutaraldehyde in Millonig's buffer (Millonig, 1961) and stored for at least 1 h in the dark at room temperature.

Nucleic acids. Calf thymus DNA (P-L Biochemicals, Inc., Milwaukee, Wisconsin) and yeast RNA (Schwarz/Mann, Orangeburg, N.Y.) were each diluted in 0.1 M-ammonium acetate and 0.01 M-MgCl₂, pH 7.0, to concentrations which provided even distributions of strands when applied for 1 min to electron microscope specimen grids (approx. 2.5 E₂₆₀ units/ml).

Electron microscopy. Samples were applied to carbon-coated copper specimen grids. After 1 min the grids were inverted and floated on distilled water for several minutes to remove salts and fixative. The grids were drained and either first dried in air or immediately stained with 1% (w/v) or 3% (w/v) uranyl acetate (UAc), unbuffered; 1% (w/v) sodium phosphotungstate (PTA), pH 6.7; or 1% (w/v) sodium zirconium glycollate (NZG), pH 7.0 (Polysciences, Inc., Warrington, Pa.).

Occasionally, grids were examined without staining or after staining with UAc and subsequent washing for 10 min with distilled water, with 0.1 M-EDTA in 0.01 M-tris buffer, pH 7.0, or with buffer alone. Some grids were shadowed with platinum-carbon at an angle of 15° under a vacuum of 10⁻⁶ Torr.

Specimens were examined in an RCA EMU-3H electron microscope at magnifications of 12000 to 55000 at 50 kV.

RESULTS

Preservation of virus components by fixation

When purified, enveloped EHV-1 was negatively stained without prior fixation or drying, envelopes usually were disrupted, and capsids frequently collapsed (Fig. 1). When fixed with OsO₄, enveloped virus particles were more homogeneous, although some envelopes were penetrated by electron-dense material. Viruses were preserved better in areas of relatively dense stain deposition.

When contrasted by shadow-casting, unfixed virus was partially flattened (Fig. 2a), whereas fixed virus produced longer, angular shadows that suggested minimal collapse and close adherence of the envelopes to the icosahedral capsids (Fig. 2b). Shadow-casting also revealed the presence of long, often branched filaments, of variable length, extending from enveloped particles (Fig. 2c), similar to those described by Smith (1963). Some characteristics of the filaments are described below.

Unfixed capsids were distorted or fragmented at the edges of negative stain pools or in areas of light stain deposition; under such conditions, capsomere sheets frequently could be seen (Fig. 3c). The appearance of disrupted, purified capsids was similar to that of broken enveloped capsids (Vernon et al. 1974) in that cracks usually originated at vertices and pentamers were frequently missing. Staining with NZG consistently resulted in fewer observations of such sheets and of fragmented capsids (Fig. 3a).

In contrast, after being fixed with either OsO₄ or glutaraldehyde, capsid samples contained
Fig. 1. Effects of OsO₄ fixation on negatively stained EHV-1 virus particles. (a) Unfixed, stained with 1% PTA. Most envelopes are disrupted, and virtually 'naked' capsids appear. (b), (c) Fixed virus particles stained with 1% PTA (b) or 1% NZG (c). Fixation has minimized envelope disruption, though some have been penetrated by stain.
Effects of OsO$_4$ on shadowed EHV-I virus particles. (a) Unfixed. Virus particles have partially collapsed. Filaments extend from the upper particle. (b) Fixed. The shadows demonstrate minimal flattening of the virus particles. Fixation increases the electron density of the particles. (c) Fixed virus particle, stained with 1% UAc, then rinsed with water. The filament, positively stained, consists of a fibrillar aggregate.

Effects of drying virus before applying stain

On the assumption that disruption occurred during the staining procedure, we investigated the effects of drying on virus particles with and without stain. Fig. 2 has already shown that when films containing unfixed enveloped virus were permitted to dry before being stained, the particles partially collapsed, and capsids were often flattened in situ. Fixation with OsO$_4$ minimized virus collapse.

All capsids, whether fixed or unfixed, collapsed in situ when dried on carbon films before being stained, but fixed particles were not fragmented (Fig. 4). The collapse of fixed capsids was prevented by staining grids with UAc before drying and shadow-casting (Fig. 4h); this observation corroborated the results of Smith & Melnick (1962a). Neither bovine serum albumin nor cytochrome c, at concentrations of 100 µg/ml, prevented collapse when mixed with capsids before their application to grids.

The combination of fixation of purified capsids and avoidance of drying before staining resulted in the almost complete absence of recognizable capsomere sheets.
Fig. 3. Effects of fixation on negatively-stained EHV-1 capsids. Grids were not permitted to dry before staining. 
(a), (c), (d) Unfixed capsids, many of which are distorted in heavily stained areas and split or fragmented in lightly stained areas. Most 'cracks' originated at capsid vertices, and pentamers frequently were missing. Degradation was observed more frequently after PTA (c) and UAc (d) staining than after NZG (a) staining. 
(b) Fixed with glutaraldehyde, stained with NZG. The particle population was more homogeneous with respect to shape and size, and capsomere sheets were only rarely seen. 
(e) Fixed with OsO₄, stained with NZG. Although the capsid population was homogeneous, the visibility of virus substructures was impaired.
Fig. 4. Effects of drying on EHV-1 capsids. Capsids were dried on carbon films before being shadowed or stained with UAc (a to c) or PTA (d, e). (a), (d), (f) Unfixed. All unfixed capsids have collapsed and split. (b) Fixed with glutaraldehyde. (c), (e), (g) Fixed with OsO₄. Fixed particles have flattened without fragmenting. (h) Fixed with OsO₄, stained with UAc before drying. Capsids were protected from flattening by the stain.
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Characterization of extra-viral filaments

Smith (1963) suggested that DNA-like filaments seen in his herpesvirus preparations had been extruded from disrupted viral cores. Our results, though not definitive, provide further evidence for that theory.

The filaments mentioned above, subsequently found to be positively stained by UAc, were first observed in preparations of shadow-cast, unfixed virus. They were seen as frequently extending from dried, fixed virus particles but not from particles stained before being dried. Stained filaments often appeared as aggregates of micro-fibrils, the thinnest of which were 2.5 nm thick (Fig. 2c). Bernhard's (1969) method for removing UAc differentially from DNA-containing cell constituents, which has been used to locate the DNA in herpes-virus cores (Furlong, Swift & Roizman, 1972), was adapted for use on samples adsorbed to carbon films. Positively-staining UAc was removed from virus filaments by flotation of the grids on 0.1 M-EDTA in tris buffer, but not on buffer alone, thus suggesting that the filaments contain DNA. We tested the differential removal of UAc by floating grids on EDTA and successfully removed the stain from previously-spread calf thymus DNA, while stain was not removed from yeast RNA.

DISCUSSION

The results indicate that two simple precautions should be followed to minimize the in situ disruption of herpesvirus envelopes and capsid shells during the negative staining procedure. The virus particles should be fixed before their application to carbon films, and drying of the films before stain is applied should be avoided assiduously.

Dehydration appears to be the major cause of in situ capsid fragmentation and envelope disruption, which frequently is sufficient to produce the appearance of sizeable numbers of 'naked' capsids. The penetration by negative stain into envelopes of herpesviruses dried on grids has been described by Watson & Wildy (1963). Our results indicate that fixation with OsO₄ minimizes both envelope disruption and particle flattening, thereby permitting more definitive evaluation of enveloped virus particles. The envelopes of herpes simplex virus type 1 also were protected by fixation (unpublished observations).

Dehydration also resulted in the fragmentation of virtually all unfixed capsids. Fixation prevented such disruption, as judged by the absence of cracked capsids and capsomere sheets. Although both OsO₄ and glutaraldehyde effectively stabilized the particles, superior ultrastructural detail was observed with the latter fixative. However, fixation did not prevent capsids from flattening during drying. Therefore, negative stain, which offers additional support to the particles, should be applied before drying. Our observations of EHV-1 capsids dried and shadowed after staining confirm the findings of Smith & Melnick (1962a) that UAc offers protection against disruption.

Envelope disruption also is visible in micrographs of virus prepared by the 'pseudo-replication' method (Smith, 1963, 1964; Abodeely, Lawson & Randall, 1970; Ludwig et al. 1971; Perdue et al. 1974), which requires the drying of samples on agar (Sharp et al. 1952). Although OsO₄ vapour has been used to fix other enveloped viruses for that method (Smith, Benyesh-Melnick & Fernbach, 1964; McCombs, Benyesh-Melnick & Brunschwig, 1966), only recently has it been employed with herpesvirus materials. However, the fixation followed drying of the virus, and the published micrographs showed disrupted envelopes (Schaffer et al. 1974).

It should be noted that naked capsids observed by the pseudoreplication method do
not seem to disintegrate as frequently as do enveloped virus particles or capsids dried on grids (Smith & Melnick, 1962a; Smith, 1963, 1964; Abodeely et al., 1970; Ludwig et al., 1971; Perdue et al., 1974). With this method the negative stain is applied, after the sample is dried, only to the pseudoreplica. It is possible that capsids dried on agar are protected from widespread fragmentation by the moisture content of the agar and by a less rigid attachment to the substrate than to carbon films. This latter possibility is substantiated by our ability to observe populations of mostly intact capsids fixed after they had been air-dried in Teflon cups or lyophilized and rehydrated with distilled water (unpublished observations).

Herpesviruses have been fixed with formaldehyde (e.g., Watson, Russell & Wildy, 1963; Bocciarelli et al., 1966; Spring & Roizman, 1967; Sydiskis, 1969), usually to stabilize virus products for density gradient centrifugation, but the resultant morphological effects on the external viral components have not been described in detail.

Employing our precautions, we have observed populations of intact capsids stored for up to 1 week in distilled water, several isotonic neutral buffers, 0.1 M-sodium acetate-acetic acid buffer, pH 4.9, or 0.1 M-sodium carbonate-sodium bicarbonate buffer, pH 10 (unpublished observations). As judged by the lack of recognizable breakdown products in preparations fixed after storage, purified capsids were stable under such conditions. Thus, electron microscopic assessments of the efficacy of subsequent degradative treatments can be made with increased confidence.

As the ultrastructural details of virus capsids varied somewhat with the negative stain employed, the use of a variety of stains for herpesvirus samples is advocated. The selective uses of PTA and UAc have been documented (Wildy et al., 1960; Smith & Melnick, 1962a, b; Smith, 1963). We have found NZG, which may act as a fixative by cross-linking molecular structures (Cohen, Garner & Lund, 1966), to be a convenient stain for both enveloped and non-enveloped herpesvirus. At neutral pH and low concentration, it spreads as well as, and is more evenly distributed over virus components than, PTA or UAc. Furthermore, it has no apparent disruptive effects on other enveloped viruses (unpublished observations), and it fails to precipitate under a wide range of salt concentrations or pH (Buckmire & Murray, 1970). However, NZG is not an ideal stain under all circumstances. It sometimes has a marked granularity at high magnifications, even when phase-contrast effects are minimal, thus sometimes making difficult clear definition of virus substructures.

We are grateful to Dr C. Long for frequent, helpful consultation, to Mr R. Hartzell for technical assistance, and to Mrs T. Schaffer for typing.

This work was supported, in part, by a U.S.P.H.S. research grant to W.C.L. from the National Institute of Allergy and Infectious Diseases, and by U.S.P.H.S. grant DE-02623 to G.H.C. from the National Institute of Dental Research.

REFERENCES


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(Received 27 October 1975)