Purification and properties of virus particles, infectious subviral particles, cores and VP7 crystals of African horsesickness virus serotype 9

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Methods were developed for the purification, at high yield, of four different particle types of African horsesickness virus serotype 9 (AHSV-9). These products included virus particles purified on CsCl gradients which contain proteins apparently directly comparable to those of bluetongue virus (VP1 to VP7); virus particles purified on sucrose gradients which also contain, as a variable component, protein NS2; infectious subviral particles (ISVPs), containing chymotrypsin cleavage products of VP2; and cores, obtained by treating purified ISVPs with 1 M-MgCl₂ to remove the components of the outer capsid layer (VP5 and VP2 cleavage products). Additional protein bands migrating with apparent Mr's lower than that of VP5 were detected during SDS–PAGE analysis of virus particles. These appear to be conformational variants of VP5 and are identified as VP5' and VP5''. BHK-21 cells infected with this strain of AHSV-9 produce large quantities of flat, usually hexagonal crystals of VP7, a major group antigen and core protein; these were also purified. Either 20 mg of virus particles, 20 mg of ISVPs or 10 mg of cores as well as 20 mg of VP7 crystals could be purified from approximately 8×10⁸ infected cells. None of the preparations of particles or crystals showed any detectable contamination with BHK-21 cell proteins or antigens, as determined by SDS–PAGE or indirect ELISA. Virus particle and ISVP preparations had specific infectivities for BHK-21 cells (approximately 1×10⁹ TCID₅₀/A₂₆₀ unit) but the infectivity of cores was approximately 10³-fold lower.

Introduction

African horsesickness is caused by an arthropod-borne virus which is recognized primarily as a pathogen of horses and other Equidae. African horsesickness virus (AHSV) is a member of the Orbivirus genus, within the family Reoviridae (Holmes, 1991) and possesses a dsRNA genome composed of 10 segments (Bremer, 1976) packaged within the core of a double-layered icosahedral protein capsid (Oellerman et al., 1970). AHSV is relatively closely related to bluetongue virus (BTV) (the prototype orbivirus) and epizootic haemorrhagic disease virus, two other serogroups of orbiviruses with which it shows some serological cross-reaction and RNA sequence homology (Huismans & Eaton, 1992; Gorman et al., 1983; Van Staden et al., 1991; Nel et al., 1992).

Previous reports on the purification and structural protein components of AHSV (Oellerman et al., 1970; Bremer, 1976; Grubman & Lewis, 1992) are all based on procedures published for BTV (Verwoerdt, 1969; Verwoerd et al., 1972; Huismans, 1979; Huismans et al., 1987a, b; Mecham et al., 1986). These methods involve fluorocarbon extraction of infected cell homogenates, followed by sucrose or caesium chloride gradient centrifugation. However, using these methods we were unable to recover significant amounts of purified virus particles of BTV serotype 1 from South Africa (BTVISA) from either sucrose or CsCl centrifugation gradients. Similar difficulties were anticipated with AHSV. The yield of AHSV particles was not reported in earlier studies and the evaluation of particle purity was limited to electrophoretic analyses of the virus proteins, followed by staining with Coomassie blue or silver, or fluorography. Using these techniques, it is relatively easy to identify the major AHSV outer capsid proteins (VP2 and VP5) and core proteins (VP3 and VP7). The identification of the minor core proteins and non-structural proteins has been less straightforward, owing to the possibility of low-level contamination with host cell or non-structural virus proteins, together with variations in the expression or labelling levels and the migration of some proteins during electrophoresis. In consequence, some confusion still exists over the identity of some AHSV proteins, which has resulted in the use of a protein nomenclature (Grubman & Lewis, 1992) that is inconsistent with that previously used for BTV, the prototype orbivirus (Gorman et al., 1985). An analysis of the genome
segment coding assignments of AHSV (R. S. O'Hara, J. N. Burroughs & P. P. C. Mertens, unpublished results) has helped us to identify the primary gene products and consequently to confirm the nature and identity of the individual virus structural proteins. AHSV particles purified by the methods described here have a protein composition essentially similar to that of BTV particles and we were therefore able to retain a protein nomenclature consistent with that for BTV and as recommended by Gorman et al. (1985).

We describe methods for the high yield purification of four different particle types of AHSV serotype 9 (AHSV-9) from infected BHK-21 cells; in addition, flat, usually hexagonal crystals of the major core protein and group antigen VP7 were purified. The purification of such crystals from orbivirus-infected cells and their characterization has not previously been reported.

Methods

Virus. AHSV-9 [South African isolate, 2 May 1973, designated 90/61 (MB3, BHK4)] and AHSV-2 (MB3, BHK2) were supplied by Dr B. J. Erasmus. They were plaque-puriﬁed three times in BHK-21 cells. The growth and partial puriﬁcation of AHSV was carried out exactly as described for BTV by Mertens et al. (1987).

Virus particles. The partially purified virus was resuspended in 10 mM-DTT and 1% sodium N-lauroyl sarcosine (NLS) until the density turbid suspension had clariﬁed (usually within 15 min), and was then centrifuged through 10 ml 40% (w/v) sucrose (containing 0.1% NLS) onto a 5 ml 50% (w/v) sucrose cushion (Beckman SW28 rotor, 2 h, 60,000 g, 4°C). The virus particles, which formed a blue opalescent band at the interface of the sucrose solutions, were recovered and dialysed against 0.1 M-Tris-HCl pH 8.0 containing 0.1% NLS. Virus particles could also be purifed using CsCl gradient centrifugation. Preformed gradients were made by carefully overlaying 2 ml of 1.39 g/ml CsCl solution with 2 ml of 1.30 g/ml CsCl solution followed by 1 ml of 40% (w/v) sucrose (all solutions in 0.1% NLS-Tris/HCl buffer). Material clariﬁed as above was layered onto these gradients and centrifuged in an SW40 rotor at 130,000 g for 1.5 h at 4°C. The virus particles (a sharp, white band at the interface of the CsCl solutions) were recovered and further puriﬁed in self-forming CsCl gradients [1.33 g/ml CsCl in 0.1% NLS (SW40 rotor, 130,000 g, > 17 h, 4°C)] at a density of 1.33 g/ml and dialysed as above.

ISVPs/VP7 crystals. The partially puriﬁed virus material was treated with 20 mg/ml chymotrypsin (Sigma) and 1% NLS for 1 h at 37°C; this caused the highly turbid suspension to increase in clariﬁcation but it did not become completely clear. This material was then centrifuged through a sucrose gradient with steps of 4 ml of saturated (i.e. 66% w/v) sucrose solution, 8 ml of 50% (w/w) sucrose and 8 ml of 40% (w/v) sucrose, all containing 0.1% NLS (SW28 rotor, 2 h, 60,000 g, 4°C). An opalescent blue band of ISVPs was recovered from the upper sucrose interface and dialysed against Tris–HCl buffer. A dense, greyish-white band was recovered at the lower sucrose interface and this was further centrifuged on preformed discontinuous CsCl gradients (density 1.24 to 1.36 g/ml, 40% sucrose, without NLS, SW40 rotor, 1.5 h, 130,000 g, 4°C). A white band was recovered from the interface of the CsCl solutions and was centrifuged on a self-forming CsCl gradient (1.31 g/ml CsCl without NLS, SW40 rotor, > 17 h, 130,000 g, 4°C). The material again formed a sharp white band, had a density of 1.31 g/ml and was composed of crystals of the major core protein, VP7. It was recovered and dialysed against Tris–HCl buffer.

Virus cores. When material which had been treated with chymotrypsin and NLS was centrifuged on CsCl gradients using the same conditions as for virus particles, a band of ISVPs was obtained with a density of 1.34 g/ml. Following dialysis to remove NLS and treatment with 1.0 M-MgCl₂, the resulting product was centrifuged on preformed CsCl gradients (density 1.30 to 1.44 g/ml CsCl, 40% (w/v) sucrose without NLS, SW40 rotor, 1.5 h, 130,000 g, 4°C). A sharp, white band of core particles was obtained with a density of 1.36 g/ml which was harvested and dialysed against Tris–HCl buffer.

Antiserum. Guinea-pig antiserum was raised against disrupted BHK-21 cells, virus puriﬁed in sucrose or CsCl, ISVPs, cores and VP7 crystals as described by Hamblin et al. (1991).

Indirect ELISA. Diluted puriﬁed antigens were adsorbed to wells of microtitre plates. The speciﬁcity and endpoint titre of each serum were then determined by titrating a twofold dilution series of the serum against antigen as described by Hamblin et al. (1990).

Specific infectivity determinations. The infectivity of different AHSV particle preparations was determined by the production of c.p.e. using a 10-fold dilution series added to microwell plates containing BHK-21 cells. The infectivity, expressed as TCID₅₀/ml, was calculated as described by Kärber (1931). From the A₅₅₀/ml of each preparation, speciﬁc infectivities could then be expressed as TCID₅₀/A₅₅₀ unit.

Electrophoretic analysis. The [³⁵S]metionine-labelled proteins present in BHK-21 cell harvests and extracts and in puriﬁed AHSV particle preparations were analysed using 11% polyacrylamide gels with a Laemmli (1970) buffer system followed by ﬂuorography (Bonner & Laskey, 1974).

Electron microscopy. Puriﬁed AHSV preparations were stained with either 2% methanamine tungstic acid pH 8.2 or aqueous uranyl acetate and examined in an electron microscope.

Results

Partial puriﬁcation of AHSV-9 from infected tissue culture material was achieved using a combination of detergent treatments (Triton X-100 and sodium deoxycholate) and discontinuous sucrose gradient centrifugation using the procedures described for BTV (Mertens et al., 1987). When this material was compared to the original tissue culture harvest, or cytoplasmic extracts (Fig. 1, lanes 1 and 2 respectively) some increase in virus particle purity was detected but signiﬁcant amounts of non-structural AHSV proteins were still present (data not shown). Final puriﬁcation of intact virus particles was achieved by treatment with DTT (10 mM) and NLS (1%), followed by centrifugation on discontinuous sucrose gradients containing 0.1% NLS. Virus particles puriﬁed in this way were free of detectable cellular proteins and the majority of the non-structural virus proteins, but did contain proteins designated VP1 to VP7, which are directly comparable in terms of relative amounts and Mr's to those present in BTV particles (Mertens et al., 1987). However, these preparations also contained protein NS2 as a variable component (Fig. 1, lane 8), sometimes in much larger amounts than in BTV particles purified using either sucrose (BTVISA) or CsCl gradients (BTV4) (Mertens et
In contrast to BTVISA or BTV3 (which are relatively unstable in high concentrations of CsCl and lose their outer capsid proteins), it was also possible to purify DTT/NLS-treated AHSV-9 virus particles by CsCl gradient centrifugation. The particles purified by this method had a density of 1.33 g/ml, and did not contain any detectable NS2 protein (Fig. 1, lane 3). Incorporation of 0.1% NLS into these CsCl gradients was found to be necessary to obtain sharp bands of purified material. Maintenance of both types of virus particle (with or without NS2) in 0.1% NLS was essential to prevent irreversible aggregation. Storage of purified virus particles at 4 °C in 0.1 M-Tris-HCl pH 8.0, 1% NLS had no detectable effect on virus infectivity over a 12 month period. However, some breakdown of VP2 was observed even after short periods of storage, leading to the detection of additional protein bands migrating between VP3 and VP5 (Fig. 1, lane 3) that were not present in freshly purified virus (Fig. 1, lane 8). In comparable preparations of BTV particles these minor bands were detectable by Western blotting using antisera to synthetic peptides derived using sequence data for VP2 (data not shown) thereby confirming their identity as VP2 breakdown products.

If the partially purified material was treated with chymotrypsin and NLS and centrifuged through a sucrose gradient, modified particles (identified as ISVPs) were recovered from the upper sucrose interface. In these particles the outer capsid protein, VP2, had been cleaved into five smaller products identified as VP2a to VP2e (Fig. 1, lane 4) which have been shown to be derived from virus structural proteins by Western blotting using antisera to purified AHSV-9 virus particles (data not shown). This contrasts with ISVPs of BTVISA in which case only three chymotrypsin cleavage products were consistently associated with the particle. Material forming a dense greyish-white band was also recovered from the lower sucrose interface and was further purified on preformed and self-forming CsCl gradients. This material, which formed a sharp white band with a density of 1.31 g/ml, had a crystalline appearance when visualized by light microscopy and was composed entirely of the major core protein and group antigen VP7 (Fig. 1, lane 7). These crystals, which were present during the early stages of virus purification, are thought to form within the infected cells but are not thought to be the product of infection with defective interfering particles, since they were also produced during replication of virus which had been plaque-purified an additional three times and during infections at both high and low multiplicities.
Table 1. Specific infectivity (TCID$_{50}$/A$_{260}$ unit) of purified AHSV-9 virus particles, ISVPs and cores

<table>
<thead>
<tr>
<th>Viral material</th>
<th>Specific infectivity</th>
</tr>
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<tbody>
<tr>
<td>Virus particles from sucrose*</td>
<td>$1.7 \times 10^9$</td>
</tr>
<tr>
<td>Virus particles from CsCl*</td>
<td>$0.5 \times 10^9$</td>
</tr>
<tr>
<td>ISVPs from sucrose</td>
<td>$0.7 \times 10^9$</td>
</tr>
<tr>
<td>ISVPs from CsCl</td>
<td>$0.9 \times 10^9$</td>
</tr>
<tr>
<td>Cores</td>
<td>$1.5 \times 10^4$</td>
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* Particles were purified and maintained in 1-0% or 0-1% NLS respectively and are equivalent to the 'disaggregated' BTV virus particles described by Mertens et al. (1987).

It was subsequently found that VP7 crystals were at least partially soluble in 1-0% NLS and 10 mM-DTT (conditions used for virus particle purification) and although the crystals could also be purified from the gradients used for final isolation of virus particles, by adding a 66% (w/v) sucrose step, they tended to be smaller, their yield was lower and they had a more rounded appearance.

When material which had been treated with chymotrypsin and NLS was centrifuged on CsCl gradients using the same conditions as for virus particles, the outer capsid was not stripped off (in contrast to BTV3 or BTVISA; Mertens et al., 1987) and a band of ISVPs was obtained at a density of 1.34 g/ml. These particles had the same protein composition as those purified using sucrose gradients (Fig. 1, lane 5).

Following dialysis to remove NLS, ISVPs were treated with 1.0 M-MgCl$_2$, resulting in their conversion to virus cores, which were then purified by CsCl gradient centrifugation at a density of 1.36 g/ml, and contained proteins VP1, VP3, VP4, VP6 and VP7 (Fig. 1, lane 6). AHSV cores appeared to be unstable in 1.5 M-MgCl$_2$, conditions known to release cores of BTV4 from the outer capsid components (Mertens et al., 1987), whereas

![Fig. 3. Indirect ELISA using twofold dilution series of normal guinea-pig sera (△) or guinea-pig antiserum raised against BHK cellular antigens (★), virus particles purified on sucrose (●), virus particles purified on CsCl gradients (○), ISVPs purified on sucrose gradients (■), cores (□) and VP7 crystals (▲). These sera were titrated against each antigen. (a) BHK cell antigen; (b) unpurified AHSV; (c) virus from sucrose; (d) virus from CsCl; (e) ISVP; (f) cores; (g) VP7 crystals; (h) A. californica NPV polyhedrin.](image-url)
at 0.5 M-MgCl₂ only a low rate of conversion to cores was obtained.

Although the amount of [³⁵S]methionine label incorporated into VP4 was low, VP4 was consistently detected in all preparations of intact and modified particles and comigrated with the primary translation product of genome segment 4 (O’Hara et al., unpublished), thereby confirming its identity as a virus structural protein. The VP5 band detected in purified virus could sometimes be resolved into two distinct bands by SDS–PAGE under standard conditions (identified as VP5 and VP5’; Fig. 1, lane 9). Purified AHSV-2 virus particles were also analysed by SDS–PAGE with decreasing concentrations of 2-mercaptoethanol in the sample buffer. At a concentration of 0.1% (v/v) another band (VP5”) running slightly faster than VP5’ was also detected (Fig. 2, lane 2); but was not seen under normal reducing conditions (Fig. 2, lane 1). As the amount of reducing agent was decreased further, from 0.01% to zero, the relative amount of VP5” increased and VP5’ disappeared. When VP5 and VP5’ were extracted from gels and compared by Cleveland mapping
they produced indistinguishable polypeptide cleavage products. In addition, when these three proteins (VP5, VP5' and VP5") were reanalysed by SDS-PAGE, under fully reducing conditions, they all comigrated with unpurified VP5 from intact virus particles (data not shown).

In a typical preparation, AHSV was purified from forty 850 cm$^3$ roller bottles of BHK-21 cell monolayers (about $8 \times 10^9$ cells). These procedures yielded approximately 50 $A_{260}$ units of particles, which is equivalent to either 20 mg of virus particles, 20 mg of ISVPs or 10 mg of cores, (assuming the same relationship between $A_{260}$ and particle concentrations as with BTV) and approximately 20 mg of VP7 crystals.

There was no significant difference in specific infectivity of virus particles or ISVPs purified using sucrose or CsCl (Table 1). However, the value for cores was approximately $10^3$-fold lower. These results are similar to those for purified particles of BTVISA and BTV4 (Mertens et al., 1987, 1993).

To confirm that all the AHSV preparations were free of contaminating BHK-21 cell antigens, antisera were raised in guinea-pigs to the different purified particles and VP7 crystals. The specificity and endpoint titres of each serum were determined by an indirect ELISA. A BHK cell antigen preparation gave a strong reaction with its homologous antiserum (Fig. 3a). Unpurified AHSV antigen gave a strong reaction with both homologous anti-virus serum and anti-BHK cell serum (Fig. 3b). However, although all of the purified AHSV

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**Fig. 5.** Electron micrographs of VP7 crystals, stained with 2% methylamine tungstate. (a) Typical preparation, (b) crystal with hexagonal profile, (c) lattice detail from hexagonal particles and (d) crystalline fragment showing edge detail. Bar marker in (d) represents 4.3 μm in (a), 260 nm in (b), 104 nm in (c) and 73 nm in (d).
African horsesickness virus purification

Effective methods have been developed for the purification of ISVPs, cores and VP7 crystals of AHSV-9. The VP7 crystal structure and the AHSV particles containing NS2 protein have not been described before. It is conceivable that NS2 is copurified with virus particles or is bound to the outer virus surface in some way rather than representing a true structural component and is released by high CsCl concentrations.

Grubman & Lewis (1992) were unable to detect either of the $[^{35}S]$methionine-labelled minor proteins (VP4 or VP6) described by Bremer (1976), or any proteins analogous to VP4 and VP6 of BTV, in AHSV particles purified using the method of Mecham et al. (1986). However, they did report a series of minor bands detected by silver staining, which they suggested included VP4 and VP6. The virus purification procedure used did not include detergent and reducing agent treatment which we have found to be essential to release virus particles from cellular materials. When Mecham et al. (1986) originally used this method for BTV, most of the preparations contained a protein of $M$, close to that of NS1, which may indicate that the method gives incomplete purification. Minor bands detected by silver staining in whole virus preparations may therefore represent contaminants rather than real structural components. Cellular proteins have been identified which migrate near VP4 during electrophoresis, are still synthesized late in infection by AHSV and are more highly labelled with $[^{35}S]$methionine than AHSV VP4 (which consistently labelled less well than VP4 of BTV) (Fig. 1, lane 1; O'Hara et al., unpublished). This may explain why Bremer (1976) reported two minor protein bands in preparations of virus particles of AHSV, VP4 and VP4a, the identity of which relative to VP4 of BTV remains uncertain. The data reported here and to be reported by O'Hara et al. indicate that by comparison with BTV, VP6 and possibly VP4 of AHSV were incorrectly identified in earlier reports.

In vitro translation of AHSV genome segment 6 (numbered according to its migration during electrophoresis on 1% agarose gels) yielded at least three related proteins, the largest of which comigrated with the major outer capsid protein VP5 during SDS–PAGE (O'Hara et al., unpublished results). Grubman & Lewis (1992) refer to the faster migrating translation product of genome segment 6 of AHSV-4 as VP6, although no evidence was presented to show that this product was equivalent to VP6 of BTV (which is encoded by genome
segment 9) and its Cleveland map analysis was similar to that of VP5. We have observed in purified virus three distinct forms of VP5 with different migration rates in SDS-PAGE (VP5, VP5' and VP5''), which after recovery and re-analysis by SDS-PAGE all comigrate and therefore appear to be of similar \( M_r \). It is considered likely that VP5 can adopt different conformational structures which are at least partially dependent on the reducing environment used and which alter its rate of migration during SDS-PAGE. Laviada et al. (1993) also detected a second protein band in the region of VP5 when purified particles of AHSV-4 were analysed by SDS-PAGE. They confirmed its relatedness to VP5 by its reaction with a VP5-specific monoclonal antibody. We have identified the different forms of VP5 as VP5', VP5'', and have identified the different major bands detected after in vitro translation of segment 6 of AHSV-9 as VP5, VP5a and VP5b (O'Hara et al., unpublished) as suggested by Gorman et al. (1985). The equivalence of the VP5a or VP5b bands to either VP5' or VP5'', if any, remains to be determined.

The nomenclature for AHSV proteins previously used by Grubman & Lewis (1992) is incompatible with that for BTV (Gorman et al., 1985; Mertens et al., 1987) and would cause confusion in Orbivirus protein nomenclature as a whole. Grubman & Lewis (1992) did not identify any other AHSV minor structural proteins which were directly comparable to VP6/VP6A found in purified particles of BTV (Mertens et al., 1984, 1987; Verwoerd et al., 1972). The minor proteins that we have consistently identified in all of the AHSV-9 particles include VP1, VP4 and VP6. No other minor protein bands were detected in the preparation of cores. These minor proteins which comigrate during SDS-PAGE with the major in vitro translation products of AHSV genome segments 1, 4 and 9 respectively (O'Hara et al., unpublished results) were also present only in infected cell lysates. In each of these respects these proteins are similar to the directly comparable minor core proteins VP1, VP4 and VP6 of BTV (Mertens et al., 1984, 1987).

The purified AHSV particles and VP7 crystals have provided a direct and indirect source of useful diagnostic reagents (both antigens and antibodies) which are free from host cell protein contamination. VP7 is a major group antigen in both BTV and AHSV (Gumm & Newman, 1982; Oldfield et al., 1990) and purified VP7 crystals have been used as a reagent for group-specific tests. Antisera raised against virus particles, ISVPs, cores or VP7 have also been used in serogroup-specific ELISAs which are suitable for the detection of both AHSV and AHSV-specific antibodies in field samples and infected tissue culture (Hamblin et al., 1991, 1992).

Disc-shaped aggregates of expressed AHSV-4 VP7 have been observed in recombinant baculovirus-infected insect cells (Chuma et al., 1992), although their fine structure was not examined. The flat, usually hexagonal crystals of VP7 produced by AHSV-9 infection of BHK-21 cells have a highly ordered hexagonal lattice consistent with a dimeric or trimeric subunit structure. X-ray diffraction studies using crystals of recombinant baculovirus-expressed VP7 from BTV have indicated that the protein is grouped in a trimeric structure (Basak et al., 1992; Hewat et al., 1992a, b) and in vitro assembly studies have demonstrated both dimer and trimer formation (Wade-Evans & Mertens, 1993). The hexagonal arrangement observed in the VP7 crystal lattice appears to have direct structural similarity to the segmented, ring-shaped capsomers that are visible on the outer surface of the AHSV core [shown here and previously reported by Oellermann et al. (1970)] and which are also composed of VP7 (Hewat et al., 1992a, b). The major inner capsid protein of rotavirus (VP6), which forms the ring-shaped capsomers on single-shelled particles, has also been shown to polymerize, under appropriate conditions, to form a hexagonal lattice (Ready & Sabara, 1987). The functional significance of the VP7 crystals, if any, remains to be determined. It is possible that these crystals represent a by-product, rather than an essential component of the AHSV replication process.

The purification methods described here represent significant improvements over those previously published for AHSV both in terms of yield and purity. They also differ from those previously published for BTV (Mertens et al., 1984) in the nature of the centrifugation conditions used for the purification of two intact virus particle forms (plus and minus NS2) and the isolation of VP7 crystals. The purified particles have provided a useful source of pure AHSV RNA for molecular studies, including gene assignment, cloning and sequencing studies. They also provide material of sufficiently high purity and in the relatively large quantities that are essential for our current crystallization and X-ray diffraction studies of the different particle types (Basak et al., 1992).

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References
