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Purification and Properties of African Swine Fever Virus

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We describe a method for African swine fever (ASF) virus purification based on equilibrium centrifugation in Percoll density gradients of extracellular virions produced in infected VERO cells that yielded about 15 ± 9% recovery of the starting infectious virus particles. The purified virus preparations were essentially free of a host membrane fraction (vesicles) that could not be separated from the virus by previously described purification methods. The purified virus sedimented as a single component in sucrose velocity gradients with a sedimentation coefficient of 3,500 ± 300S, showed a DNA-protein ratio of 0.18 ± 0.02 and a specific infectivity of 2.7×10^7 PFU/μg of protein, and remained fully infectious after storage at −70°C for at least 7 months. The relative molecular weights of the 34 polypeptides detected in purified virus particles ranged from 10,000 to 150,000. Some of these proteins were probably cellular components that might account for the reactivity of purified virus with antiserum against VERO cells.

African swine fever (ASF) is an important disease of domestic pigs (47, 49). It is caused by an icosahedral cytoplasmic deoxyvirus with lipid envelopes (2, 8, 10, 18, 34, 35) and double-stranded DNA of about 170 kilobase pairs (3, 16).

ASF virus multiplies in porcine monocytes and macrophages (9, 11, 13, 32) and, after adaptation in vitro, in several established cell lines from pigs (20, 31) and other animal species (15, 17, 36, 43, 46).

The published methods for ASF virus purification have used extracellular or cell-associated, adapted virus as starting material. They include fluorocarbon extraction (26), isoelectric (42) and polyethylene glycol (16) precipitation, equilibrium centrifugation in sucrose (46) and sucrose-cesium chloride mixtures (39), hydroxyapatite treatment and potassium tartrate centrifugation (24), and incubation with Tween 80 followed by sucrose centrifugation in the presence of 1 M NaCl (6, 41). None of these reports gave the controls necessary to ascertain the purity of the final preparations, and in our hands, the methods produced variable recoveries of infectious virus particles (usually less than 1% of the starting infectious units) and heavy contamination of the final virus preparation with membrane fragments (vesicles).

Some herpesviruses have been purified in Percoll density gradients with a good yield of infectious particles (23, 37). Percoll is a mixture of polydisperse colloidal silica particles (hydrodynamic diameter ca. 30 nm) coated with polyvinylpyrrolidone, which gives density gradients of low osmolarity and viscosity after centrifugation. These properties make Percoll very useful for purifying structures that are sensitive to high ionic strength and osmolarity (37, 38).

This paper shows the use of Percoll for purifying extracellular ASF virus particles produced in VERO cells with efficient removal of contaminant vesicles and better recovery of infectious particles than that obtained by other, previously published methods. The purified virus particles were analyzed for proteins and DNA and for the presence of host cell antigens.

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MATERIALS AND METHODS

Virus and cells. The strain of ASF virus adapted to grow in VERO cells (BA71-V) has been described elsewhere (15; A. Sanz, B. García-Barreno, M. L. Nogal, E. Viñuela, and L. Enjuanes, submitted for publication). Virus infectivity was determined by plaque formation in VERO cells (15). VERO cells were obtained from the American Type Culture Collection (ATCC CCL81). They were grown at 37°C in Dulbecco modified Eagle medium with 10% newborn calf serum and passaged at weekly intervals. The cells used for virus production were passaged fewer than 12 times. Virus, cells, and culture media were tested for mycoplasma and bacterial contamination by standard methods (1).

Radioactive labeling. VERO cells were cultured in roller bottles (500 cm^2) in 50 ml of Dulbecco modified Eagle medium with 10% newborn calf serum. The medium was removed when the cultures reached a density of about 10^5 cells per cm^2, and the cells were infected with ASF virus at a multiplicity of infection of about 0.5 infectious particles per cell in 5 ml of Dulbecco modified Eagle medium with 2% serum. After 2 h of adsorption, the medium was removed and fresh medium with 2% serum (50 ml per bottle) was added to the infected cells, which were incubated until extensive cytopathic effect was observed (about 3 days after infection). DNA was labeled with 20 μCi of [^{3}H]thymidine (46 Ci/mmol; The Radiochemical Center, Amersham, England) per ml, added to the medium after virus adsorption. Proteins were labeled by addition of 20 μ Ci of [^{35}S]methionine (1,200 Ci/mmol; The Radiochemical Center) per ml or 5 μCi of a mixture of ^{14}C-amino acids (average specific activity, 295 μCi/μmol; batch 80, The Radiochemical Center) per ml to the culture medium. The concentration of unlabeled methionine or of amino acids in the culture medium was reduced 10-fold, respectively. This amino acid concentration did not change either the kinetics of virus appearance or the final virus production. Uninfected cells were labeled with 20 μCi of [^{35}S]methionine per ml for 8 h, as before. The culture medium was then removed, and the cells were washed and incubated with normal medium for 4 h before virus infection. The chase period was long enough for

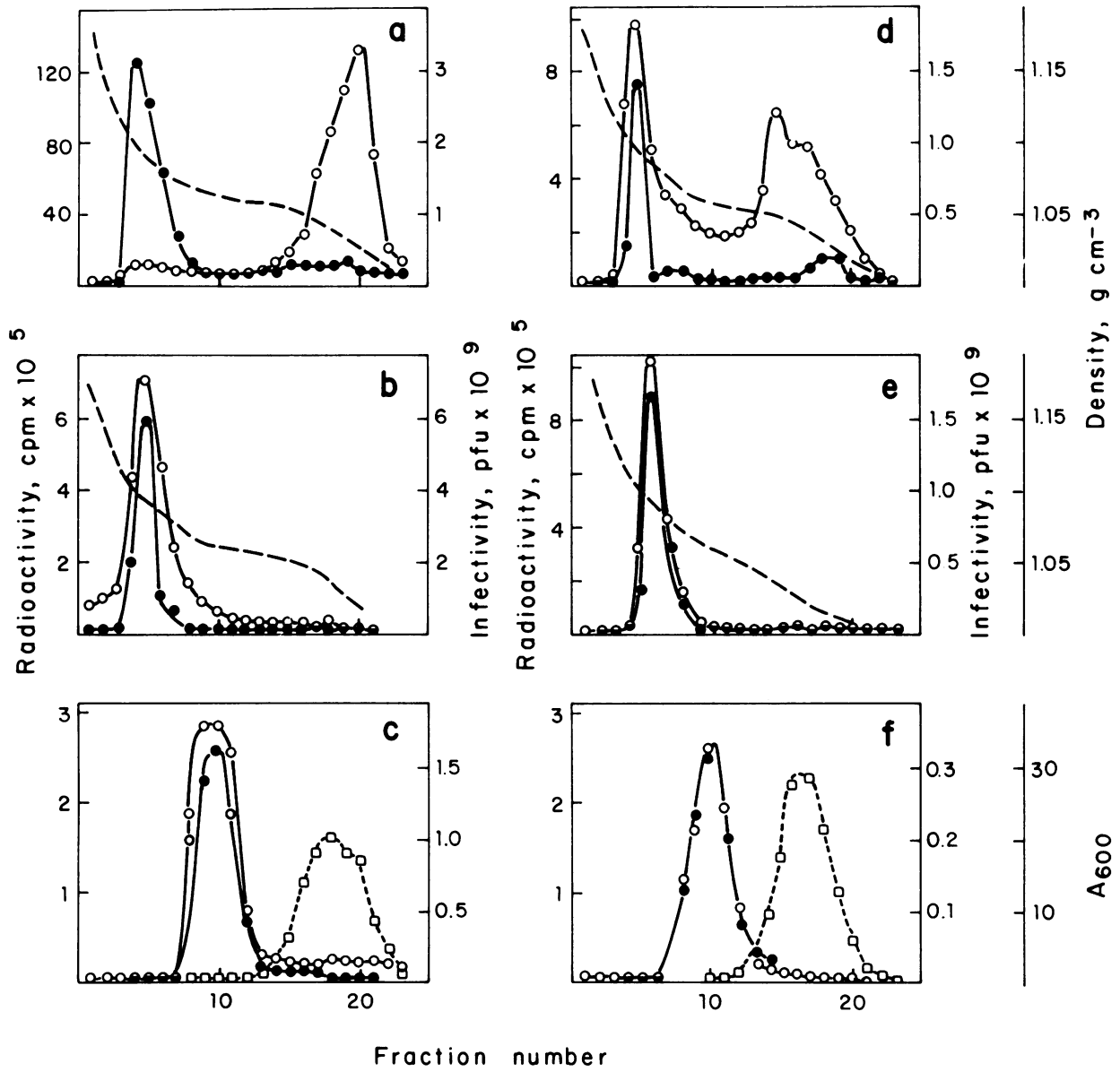

FIG. 1. Purification of extracellular ASF virus by Percoll sedimentation. VERO cells from 20 roller bottle cultures were infected with ASF virus, and one bottle was labeled with either [^{35}S]methionine (a through c) or [^{3}H]thymidine (d through f). The virus from clarified culture medium was concentrated and centrifuged on a first Percoll density gradient (a and d). The fractions containing the virus were pooled and subjected to a second Percoll density gradient step (b and e). Percoll in the virus band was removed by gel filtration through a Sephacryl S-1000 column (c and f). Portions from each fraction were taken to determine density (– –), infectious virus titers (●), total radioactivity (○), and Percoll content (□).

total incorporation of the acid-soluble radioactive components into acid-insoluble material.

Virus purification. The culture medium from ASF virus-infected VERO cells was centrifuged at low speed to remove cell debris, and the extracellular virus in the supernatant was concentrated by centrifugation in a Sorvall GS3 rotor at 8,500 rpm for 6 h at 4°C. The pellets were suspended in phosphate-buffered saline (PBS) without divalent cations. Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) was added to the virus suspension to a final Percoll concentration of 45% in PBS. The suspension was centrifuged in an angled-head T865 Sorvall rotor at 20,000 rpm for 30 min at 4°C. The gradient was fractionated from the bottom, and portions from each fraction were taken to determine density by refraction index measurement, infectivity by plaque titration, and total radioactivity by scintillation counting. The fractions containing the virus (ρ = 1.095 g/ml) were pooled and placed in the bottom of a centrifuge tube, which was filled with 45% Percoll in PBS. After centrifugation under the same conditions used for the first Percoll gradient, the virus band was recovered, and the Percoll was removed by gel filtration of the sample through a column with superfine Sephacryl S-1000 (Pharmacia) equilibrated with 0.25 M sucrose–10 mM Tris-hydrochloride (pH 7.4) at 4°C at a flow rate of 20 cm/h (21). The position of the Percoll in the effluent of the Sephacryl S-1000 column was assayed by

TABLE 1. Purification of extracellular ASF virus labeled with [^{35}S]methionine[a]

Purification step	Acid-insoluble radioactivity (10^6 cpm)	Protein (mg)	Infectivity (10^9 PFU)
Low-speed centrifugation	213.0		20.0
High-speed centrifugation	51.8	35.3	27.0
Virus, Percoll I	1.6		6.3
Virus, Percoll II	0.8		9.3
Virus, Sephacryl	0.5	0.2	5.5
Vesicles, Percoll I	14.9		0.6
Vesicles, Sephacryl	4.7	2.3	0.3

[a] Virus was obtained from 20 roller bottles which contained about 10^9 ASF virus-infected cells.

turbidity measurement at 600 nm in a portion of each fraction in 10% trichloroacetic acid. The virus in the effluent was frozen at −70°C.

The material which banded in the first Percoll gradient as a wide peak at a density of ca. 1.050 g/ml (vesicles) was filtered through Sephacryl S-1000 as described above to remove the Percoll.

Electron microscopy. (i) Negative staining. The samples were fixed with 2% glutaraldehyde in PBS for 5 min at room temperature, centrifuged on the grid of a Beckman Airfuge EM-90 electron microscopy particle-counting rotor, and stained with 2% sodium phosphotungstate, pH 7.

(ii) Thin sections. Pellets of virus or vesicles were fixed with 1% glutaraldehyde for 1 h at room temperature. The material was fixed and contrasted with 2% osmium tetroxide, dehydrated with acetone, and embedded in Vestopal-W (5). Thin sections were stained with uranyl acetate and lead citrate as described by Reynolds (40).

The samples were examined in a JEOL 100B electron microscope equipped with an anticontamination device.

DNA and protein analysis. The DNA content of ASF virus particles was determined fluorometrically (22) after treatment of the samples with Sarkosyl and proteinase K as described by Almendral et al. (3). Protein content was determined by the method of Lowry et al. (30), with bovine serum albumin as the standard.

Velocity sedimentation. [^{35}S]methionine-labeled ASF virus, purified by Percoll centrifugation, was mixed with phage T4, and the mixture was centrifuged through a 16.5-ml linear 5 to 20% sucrose gradient in PBS for 15 min at 4°C in a Sorvall AH-627 rotor at 15,000 rpm (33). The position of the marker phage T4 was determined by plaque titration on *Escherichia coli* C600.

Polyacrylamide gel electrophoresis. Polypeptides were dissociated by heating the sample in a buffer which contained 0.04 M Tris-hydrochloride (pH 6.3)–5% β-mercaptoethanol, 2.3% sodium dodecyl sulfate, and 10% glycerol (dissociation buffer) for 2 min in boiling water. Electrophoresis was carried out in 7 to 20% polyacrylamide gels by the method of Laemmli (25) in the presence of marker proteins of known molecular weight. Radioactive protein bands were detected by autoradiography of 2,5-diphenyloxazole-embedded dried gels (7). Unlabeled polypeptides were stained with silver nitrate (4).

Antisera and immunoprecipitation. (i) Antisera. Two milligrams of protein from a homogenate of either uninfected or ASF virus-infected VERO cells in Freund complete adjuvant were injected intradermally into rabbits. After 41 and 54 days, the rabbits received 1 mg of protein in Freund incomplete adjuvant, and 4 days later serum was prepared from defibrinated blood. Serum against ASF virus particles, purified by Percoll centrifugation, was prepared from rabbits that had received intramuscular injections of 50 μg of virus particles in Freund complete adjuvant. After 20 and 37 days, the rabbits received 50 μg of virus in Freund incomplete adjuvant intradermally and 25 μg of virus intravenously, respectively, and 7 days after the last injection serum was prepared from defibrinated blood. The titers of the sera against uninfected VERO cells, ASF virus-infected VERO cells, and ASF virus particles were 800, 2,200 and 50,000, respectively, as determined by an enzyme-linked immunosorbent assay (48).

(ii) Virus immunoprecipitation. Purified ASF virus particles were incubated for 60 min at 4°C with serial dilutions of either anti-VERO or anti-ASF virus serum in preimmune serum, and then half of each sample was incubated with 3% *Staphylococcus aureus* cells (Cowan I strain; Pansorbin, Calbiochem, San Diego, Calif.) in PBS and the other half was incubated in PBS alone for 30 min at 4°C. The total virus titer and the virus titer in the supernatant after low-speed centrifugation were determined by plaque formation on VERO cells.

RESULTS

Purification of extracellular virus. Extracellular ASF virus particles, produced in VERO cells in the presence of [^{35}S]methionine or [^{3}H]thymidine, were concentrated by centrifugation and sedimented to equilibrium in a Percoll gradient (Fig. 1). The infectious virus particles banded in a single peak (fractions 4 through 6) with an average density of 1.095 g/ml, which overlapped with a small, diffuse band of material labeled with radioactive methionine (Fig. 1a) or a sharp band of material labeled with radioactive thymidine (Fig. 1d). A second broad band (fractions 15 through 20), close to the top of the gradient, contained most of the radioactive proteins but no infectious virus (Fig. 1a and d). After a second equilibrium centrifugation in Percoll of the pooled fractions containing the infectious virus (Fig. 1b and e), the virus was obtained free of Percoll by gel filtration through Sephacryl S-1000 (Fig. 1c and f). Table 1 shows the recovery of infectious particles, radioactive material, and protein at the different purification steps of the ASF virus particles and vesicles labeled with [^{35}S]methionine. The specific infectivity of the virus and vesicle fractions after the last purification step was 2.7×10^7 and 1.3×10^5 PFU/μg of protein, respectively.

Electron micrographs of negatively stained and thin-sectioned ASF virus particles (Fig. 2A and B) and vesicles (Fig. 2C and D) purified by Percoll sedimentation indicated that the purified virus was essentially free of contaminants.

The recovery of infectious virions was 15.2 ± 8.8% (12 experiments), and the DNA-protein ratio was 0.18 ± 0.02 (three experiments). The corresponding values for sucrose-purified virions (6, 41) were 0.7 ± 0.3% (seven experiments) and 0.04 ± 0.006 (three experiments).

ASF virus purified by Percoll centrifugation and stored at −70°C in 0.25 M sucrose–10 mM Tris-hydrochloride (pH 7.4) retained more than 80% of its infectivity after 7 months. After 40 days at 4°C, the infectivity decreased about 100-fold in the absence of serum and about 10-fold in 10% calf serum. At 37°C, the infectivity decreased about 30-fold after 1 h in the absence of serum, but the infectious virus was stable in the presence of 1 mg of bovine serum albumin per ml in PBS.

Sedimentation coefficient of ASF virus particles. Figure 3

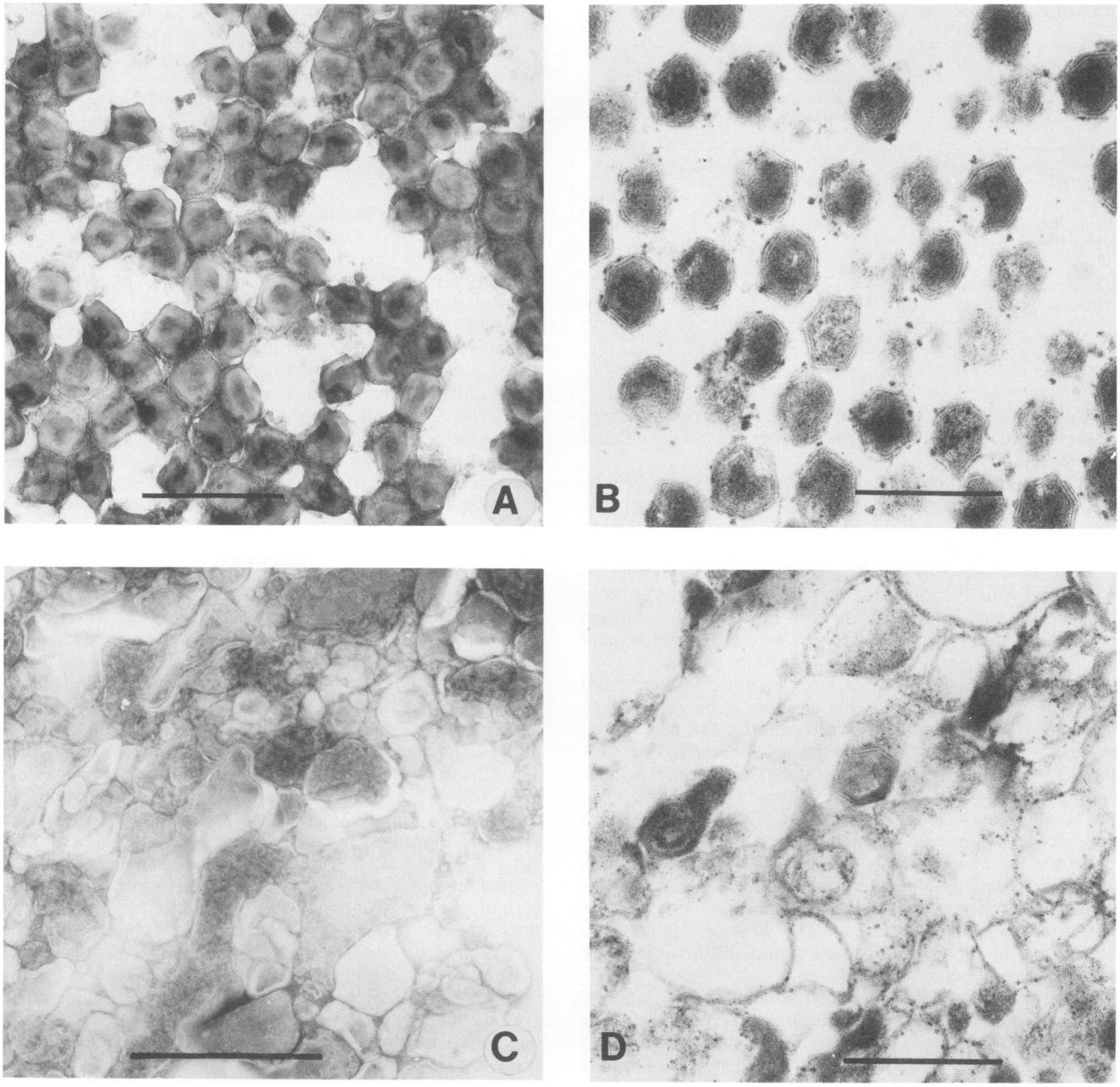

FIG. 2. Electron micrographs of Percoll-purified extracellular ASF virus particles and vesicles. Negative staining of virus (A) and vesicles (C) concentrated in an Airfuge, and thin sections of high-speed-concentrated material from virus (B) and vesicles (D). Bars, 0.5 μm.

shows the sedimentation rate of ASF virus particles purified by Percoll centrifugation in a 5 to 20% sucrose gradient. The infectious virus sedimented as a single component which overlapped with most of the material labeled with [^{35}S]methionine. Assuming that the gradient was isokinetic, the sedimentation coefficient of ASF virus particles was 3,500 ± 300S (three determinations).

Structural proteins. The electrophoretic profiles of the radioactive proteins present in virus particles (Fig. 4a and c) and vesicles (Fig. 4b and d) labeled with [^{35}S]methionine and purified by Percoll sedimentation were determined. A similar profile was observed when a mixture of ^{14}C-amino acids was used (data not shown). Also shown are the virus (Fig. 4e) and vesicle (Fig. 4f) polypeptides stained with silver nitrate after polyacrylamide gel electrophoresis.

A comparison of the radioactive polypeptides present in purified virus particles and vesicles obtained from cells labeled after (postlabeled cells; Fig. 4a and b) and before (prelabeled cells; Fig. 4c and d) the infection indicated the following.

(i) The virus particles produced in postlabeled cells contained some proteins not present in those obtained from prelabeled cells (Fig. 4a and c). These proteins, which are the most likely candidates for ASF virus structural proteins, are underlined in Table 2; the major ones were a 150,000-molecular-weight protein (p150), p130, p72, p37, p35, p17, p12, and p10.

(ii) The virus produced in postlabeled cells contained some protein bands that were less intense than those present in virus from prelabeled cells (Fig. 4a and c). These proteins

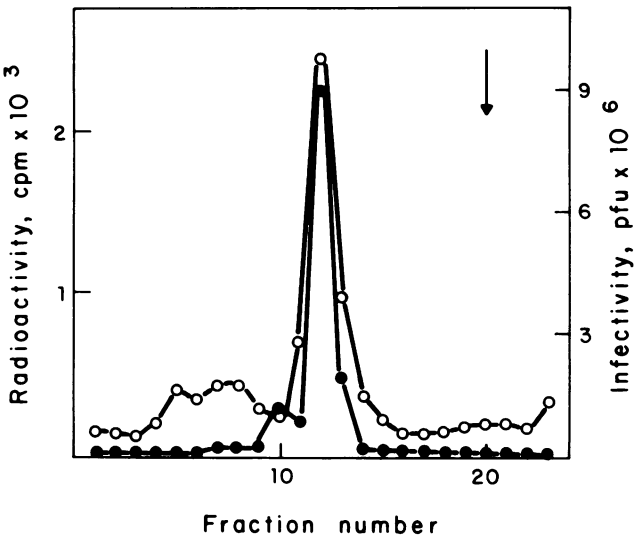

FIG. 3. Velocity sedimentation of ASF virions. Percoll-purified, [^{35}S]methionine-labeled ASF virus particles were centrifuged in a linear 5 to 20% (wt/vol) sucrose gradient in PBS. Fractions were collected from the bottom and analyzed for acid-insoluble radioactivity (○) and virus infectivity (●). Arrow, Position of marker phage T4.

could be host proteins which either were incorporated into the virions or contaminated the virus preparations or both. Proteins with these characteristics were p85 and p45, among others.

(iii) The protein composition of vesicles from prelabeled and postlabeled cells was similar except for the presence of the major viral structural proteins p150, p72, p17, and p12 (Fig. 4b and d) in the vesicles obtained from postlabeled cells.

Host antigens in purified ASF virions. The presence of cellular antigens in purified virus preparations was tested by an enzyme immunoassay with rabbit antiserum against uninfected VERO cells. The antiserum reacted with virus particles and vesicles as well as with uninfected VERO cells (data not shown). We tested for the presence of cell antigens incorporated into the virions by immunoprecipitating the infectious particles with rabbit antiserum against either purified virions or uninfected VERO cells. In the presence of *S. aureus* cells, the infectious particles were precipitated by anti-VERO serum as well as by serum against purified virus (Fig. 5), indicating that either infectious ASF virions incorporated host components or some viral components shared epitopes with host antigens.

DISCUSSION

Extracellular ASF virus particles, produced in VERO cells and concentrated by centrifugation, were separated by equilibrium sedimentation in a Percoll gradient from a noninfectious fraction that contained most of the protein (Fig. 1, Table 1) in membrane fragments (vesicles), which sometimes enclosed virus particles (Fig. 2D). Previous methods for the purification of ASF virus, based on the use of sucrose gradients (6, 41, 45), did not separate the vesicles from the virus particles.

The Percoll purification method produced a virus preparation in which about 99% of the identifiable structures observed in thin sections were virus particles (Fig. 2B); about 8% of the material could not be identified. The tests of homogeneity carried out by electron microscopy of negatively stained preparations of the material adsorbed directly to the grid were unreliable, since virus samples highly contaminated with membranes and vesicles, as seen in thin sections, appeared to be almost homogeneous when they were examined by negative staining (data not shown). This indicated a preferential binding of the virions to the grid. Nevertheless, if the samples were negatively stained after Airfuge centrifugation on the grid (Fig. 2A), the percentage of vesicle contamination in Percoll-purified virus fractions was similar to that obtained in thin-sectioned virus preparations (Fig. 2B), and less than 1% of the structures remained unidentified.

The purified ASF virus particles sedimented as a single component in an isokinetic sucrose gradient at a rate of 3,500 ± 300S (Fig. 3). In contrast, ASF virus particles partially purified in sucrose-CsCl gradients (39) sedimented in the analytical ultracentrifuge within a range of 1,800 to 3,200S (45). It is likely that this heterogeneity was due to partial degradation of the particles during purification.

The ionic strength and osmolarity of Percoll make it very useful for purifying fragile structures, and the Percoll method allowed the recovery of about 20 times more infectious ASF virus particles than could be obtained by sucrose centrifu-

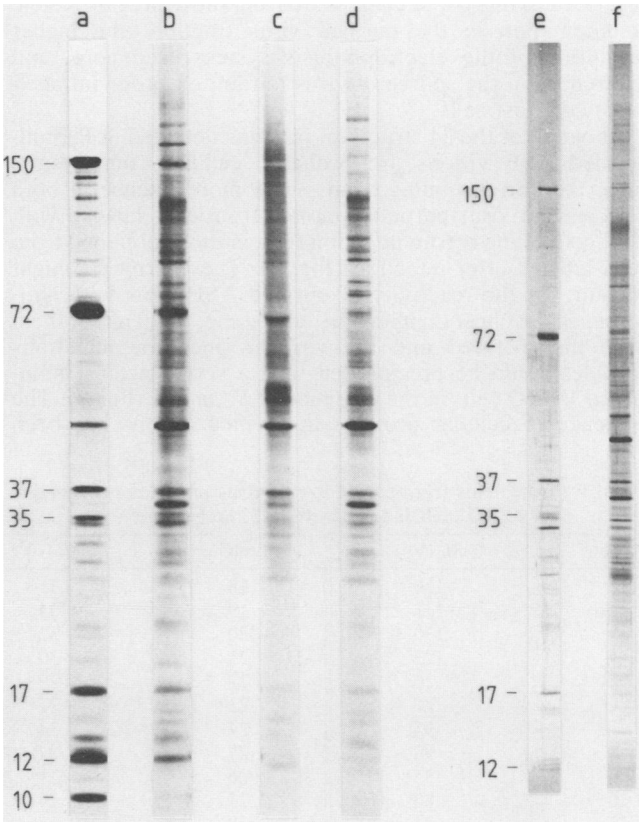

FIG. 4. Polyacrylamide gel electrophoresis of Percoll-purified ASF virus (a, c, and e) and vesicles (b, d, and f) obtained from unlabeled cells (e and f) and from cells labeled with [^{35}S]methionine after (a and b) or before (c and d) infection. An equal amount of label was loaded in each lane. The specific radioactivities of the samples were 7,541, 11,631, 2,143 and 9,657 cpm/μg of protein in lanes a, b, c, and d, respectively. Radioactive and unlabeled protein bands were detected by fluorography and silver staining, respectively. Lanes a through d and lanes e and f were from two different gels. Molecular weights (in thousands) are indicated.

gation. The separation of most of the Percoll particles from the virions could be achieved by gel filtration on superfine Sephacryl S-1000 (Fig. 1c and f). The small amount of Percoll contamination seen in some virus fractions (Fig. 2A and B) did not interfere with the chemical and biological assays shown in this work.

The DNA-protein ratio in Percoll-purified virus preparations was 0.18 ± 0.02, higher than the 0.04 ± 0.006 ratio obtained for sucrose-purified virus. This difference was mainly due to poor separation of virus particles from vesicles by sucrose centrifugation. As ASF virus contains a single DNA molecule of about 170 kilobase pairs (3, 16), the ratio of physical to infectious particles in the purified virus fraction was 54 ± 27 (mean of 10 preparations).

After polyacrylamide gradient gel electrophoresis, we detected 34 radioactive protein bands in extracellular ASF virus particles, with molecular weights ranging from 10,000 to 150,000 (Fig. 4a). A similar profile was obtained when the proteins were stained with silver nitrate (Fig. 4e). Proteins larger than 150,000 molecular weight that have been found in intracellular ASF virus purified by sucrose sedimentation (44) were not detected in preparations of extracellular virus purified by Percoll sedimentation, but appeared as components of the vesicle fraction. The discrepancy between the number and the apparent molecular weights of the ASF virus protein bands described previously (6, 27, 44) and those described in this paper could arise from the degree of vesicle contamination in the purified virus fraction, the higher resolution of the electrophoresis system used here, and differences in the source of virus (strain, extra- or intracellular) and host cells.

About 12 of the 34 structural proteins detected in Percoll-purified ASF virions are probably cellular components, since the corresponding bands were more intense in both vesicles and virus particles purified from cells labeled with [^{35}S]methionine before infection than in those obtained from cells labeled after infection (Fig. 4). These proteins might account for the reactivity of purified ASF virus with antiserum against host components, and some, if not all, of them were incorporated into the virions, since the infectious particles could be precipitated with a serum against uninfected VERO cells in the presence of *S. aureus* (Fig. 5). The presence of cellular proteins in purified virions has been described for several viruses, corresponding in some cases to host major histocompatibility or tumor-specific antigens (12, 28) that might be selectively incorporated in the process of budding through the host cell membrane (29). The biological significance of the presence of these cellular components in the virus particles is unknown, although a possible role in limiting virus infection by a response directed against these proteins has been suggested (19). It will be interesting to identify those components in the ASF virus particles and their possible relationship with the peculiar ASF virus-induced immune response (14).

TABLE 2. Proteins from purified ASF virus particles produced in VERO cells labeled with [^{35}S]methionine[a]

Protein	M_r (10^3)	Protein	M_r (10^3)
1	150	18	35
2	135	19	34
3	130	20	32
4	125	21	30
5	120	22	29
6	110	23	27
7	95	24	24
8	90	25	22
9	85	26	21
10	80	27	19
11	74	28	17
12	72	29	15
13	62	30	14
14	56	31	13
15	52	32	12
16	45	33	11
17	37	34	10

[a] The underlined proteins are the most likely candidates for ASF virus structural proteins.

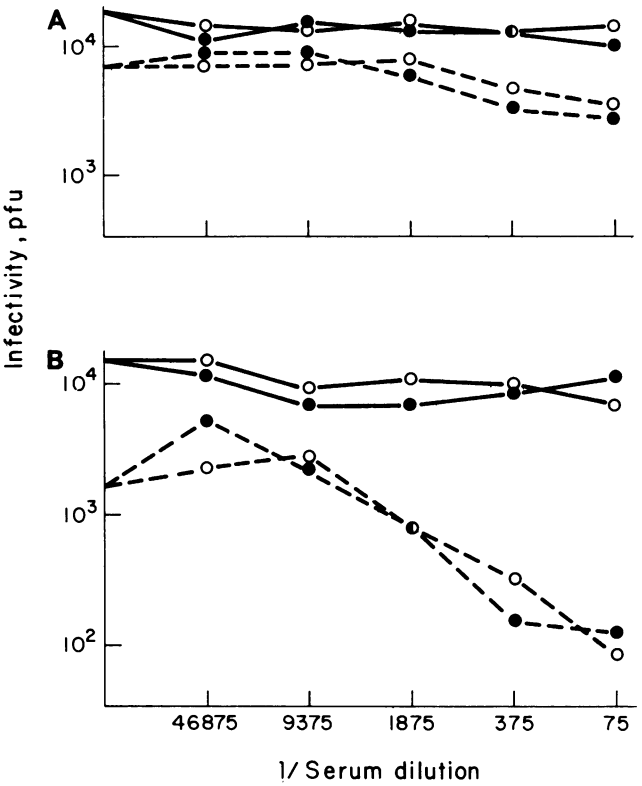

FIG. 5. ASF virus immunoprecipitation by anti-VERO (○) and anti-ASF virus (●) serum without (A) and with (B) fixed *S. aureus* cells. Total virus (———) and virus remaining in the supernatant after low-speed centrifugation (– – – –) were assayed by plaque formation on VERO cells.

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