JOURNAL OF BACTERIOLOGY, June, 1965
Vol. 89, No. 6

Fine Structure of the Coat and Nucleoid Material of Fowlpox Virus

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Received for publication 15 January 1965

ABSTRACT

HYDE, JAMES M. (University of Mississippi School of Medicine, Jackson), LANELLE G. GAFFORD, AND CHARLES C. RANDALL. Fine structure of the coat and nucleoid material of fowlpox virus. J. Bacteriol. **89**:1557–1569. 1965.—Several morphological forms characteristic of the poxvirus group were demonstrated for fowlpox virus with neutral phosphotungstic acid (PTA). Viral particles (purified from viral inclusion bodies) stained with uranyl acetate (UA) and shadowed with platinum were shown to have an external knobby surface not evident with PTA. The external coat of freshly purified viral particles seemed intact, but as the preparation aged, it appeared to unwind, resulting in twisted "rope-like" structures. This process was facilitated by use of 1% trypsin, and three dense fibrils were identified with UA within the partially detached viral coat. Studies with alkaline PTA (*p*H 9) were interpreted as revealing a complex nucleoid, but solutions above this *p*H damaged the particles. The morphology of the nucleoid was better depicted in ultrathin sections of whole virus which, when stained with UA, revealed dense coiled threads. Treatment of virus with sodium lauryl sulfate exposed an underlying coat consisting of small subunits approximately 40 A in diameter. Of great interest was the demonstration that the detergent removed strands of deoxyribonucleic acid (DNA) from the virus without destroying the contour of the particle. The origin of the strands was definitely the fine uranophilic, coiled threads of the nucleoid, which probably represent the DNA molecule(s). That the extracted material was largely DNA was proved by digestion with deoxyribonuclease and resistance to ribonuclease and trypsin. These studies illustrate how a variety of electron microscopic techniques may be utilized alone or in combination to reveal hitherto undescribed fine structure of viral particles.

This laboratory has been concerned for a number of years with the study of fowlpox virus (FP). Previous investigations have been concerned with the fine structure and development of the virus in infected tissues (Arhelger et al., 1962; Arhelger and Randall, 1964).

In the present report, the fine structure of isolated virus was analyzed by a variety of electron microscopy techniques, and similarities to other poxviruses are pointed out. In other instances, new information concerning the architecture of FP is elucidated, including the exposure of deoxyribonucleic acid (DNA) by detergent extraction methods. The present work was reported in preliminary form by Hyde, Gafford, and Randall (1964).

MATERIALS AND METHODS

Virus and purification from inclusions. Most of this study was conducted with FP derived from chick scalp. This strain of virus, the method of infecting chick scalp, the isolation and purification of viral inclusions, and the subsequent preparation of highly purified virus from sonic-treated inclusions have been described (Randall, Gafford, and Darlington, 1962). The virus particles isolated by these techniques were morphologically homogeneous as judged by electron microscopy, as will be described.

The virus strain was easily adapted to chick embryo monolayer cultures, and virus from this source will be referred to where appropriate.

Pseudoreplicas and staining techniques. Virus suspensions were prepared for electron microscopy according to the pseudoreplica technique of Sharp (1960). Briefly, 2% agar was dissolved in 0.9% saline and poured into petri dishes to a depth of approximately 4 mm. Squares (1 cm) were cut from the solidified agar and placed on microscope slides near one end. The surface of an agar square was then coated with a drop of the virus suspension and allowed to dry. The agar was flooded with purified 1% collodion in amyl acetate and drained on bibulous paper until dry, and the edges of the agar were carefully trimmed with a razor blade to free the adherent collodion from the

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slide. This procedure left the agar square with its upper surface coated with the virus suspension, covered with a very thin layer of collodion. Staining solutions of 0.5 or 0.007% uranyl acetate (UA) and 0.5% potassium phosphotungstate (PTA) at *p*H 7 were placed in separate petri dishes, and the collodion membranes with adhering virus particles were floated off the agar onto these solutions, as described by Smith and Melnick (1962) and Rhim, Smith, and Melnick (1961). Nickel specimen grids (200 mesh) were dropped onto the floating films, and the specimens were picked up on brass pegs, drained by touching one edge to bibulous paper, allowed to dry, and examined in either an RCA model EMU-3F or EMU-3G electron microscope.

Fixation of virus and preparation of thin sections. Suspensions of purified virus were fixed for 30 min at 4 C in 1% buffered osmium tetroxide (Palade, 1952), and then centrifuged for 30 min at approximately 10,000 × *g*. The supernatant fluid was discarded, and the virus pellet was embedded in two drops of 3% agar at 50 C and cooled quickly in an ice bath. The agar button was removed from the centrifuge tube, cut into 1-mm sections, rapidly dehydrated in graded alcohols, and embedded in EPON-812 (Luft, 1961). Thin sections were cut on a Servall Porter-Blum model MT-1 ultramicrotome with glass knives. The sections were mounted on uncoated 200-mesh copper specimen grids, stained for 30 min with 2% UA, and examined in the electron microscope.

Trypsinization of whole virus. A suspension of FP was mixed with an equal volume of filtered 1% trypsin (Difco) in Sorensen's phosphate buffer (*p*H 7.6), and incubated at 37 C for 30 min. Immediately following the incubation period, pseudoreplicas of the trypsinized virus were prepared for electron microscopy.

Treatment of whole virus with sodium lauryl sulfate (SLS). Purified SLS (USP grade; Fisher Scientific Co., Pittsburgh, Pa.) was mixed in buffer containing 0.02 M sodium citrate and 0.15 M sodium chloride to give a 4% solution. An equal volume of virus suspension was mixed with a sample of SLS solution, and the mixture was stirred gently. Pseudoreplicas were prepared from this suspension for examination, including enzymatic digestion, as described below.

Treatment of pseudoreplicas with nucleases and trypsin. Deoxyribonuclease I (once crystallized; Worthington Biochemical Corp., Freehold, N.J.) was mixed in 0.02 M phosphate buffer and 0.005 M $MgSO_4$ at *p*H 7.6. Ribonuclease (five times crystallized, salt free; Mann Research Laboratory, New York, N.Y.), was dissolved in 0.02 M phosphate buffer, *p*H 7.6. Trypsin (twice crystallized; Worthington Biochemical Corp.) was also dissolved in the *p*H 7.6 phosphate buffer. The final concentration of all three enzymes was 100 μg/ml. A drop of enzyme solution was added to the dry surface of an agar square, to which SLS-treated virus was adsorbed, and allowed to act at room temperature for 15 min. The agar was then flooded with purified collodion solution, and pseudoreplicas were prepared. Other pseudoreplicas of the SLS-virus mixture were treated with enzymes or suitable control solutions at 37 C for 30 to 60 min.

RESULTS

Surface structure studied with neutral PTA and purified virus. Figure 1 shows virus stained with 0.5% PTA at *p*H 7. It is evident that PTA has penetrated the virus particles to some extent, rendering them relatively electron dense. A peripheral capsularlike structure is conspicuous, although external surface structure is not. The general appearance (without thread structure) is similar to the type 2 form of vaccinia virus (Nagington and Horne, 1962). This type of particle was referred to by Dales (1962), on morphological grounds, as damaged or empty. Similar forms were termed "B" by Noyes (1962*a*, *b*) and "C" by Westwood et al. (1964). The terminology of Westwood will be used in this report.

Combined uranyl acetate staining and platinum shadowing. C forms of fowlpox particles stained with 0.007% UA appeared to have a knobby surface. Light platinum shadowing of UA-stained virus greatly enhanced the contrast of these surface units (Fig. 2). The knobs were irregularly arranged, varying in size from 300 to 400 A, with indications of a dense central core. Figure 2 also demonstrates the tendency of the surface layers to coalesce.

Suspensions which had been stored for several weeks or more required sonic treatment (prior to use) for 30 sec in a 50-w, 9-kc Raytheon sonic oscillator to obtain sufficient dispersion for electron microscopy. The appearance of these particles was the same whether freshly isolated or stored and sonically treated, except that in older preparations free strands of viral material were observed. Probably as the result of efforts to disperse the virus, strands became increasingly noticeable in the form of "rope-like" structures with virus particles attached at various points (Fig. 3A and B). Figure 3C shows a portion of the "frayed" end of one of these "ropes." The subunits within a strand measure the same as the knobs on the intact virus (Fig. 2 and 3) and appear to contain a core of dense material.

Neutral PTA and impure virus. A form of poxvirus with threadlike peripheral structure has been described by Herzberg et al. (1961). This particle is apparently identical to type 1 of Nagington and Horne (1962) and type M of Westwood et al. (1964). Early attempts in this laboratory to obtain the M form of FP met with failure. Hence, the gentlest methods available were used to obtain virus samples. Virus was

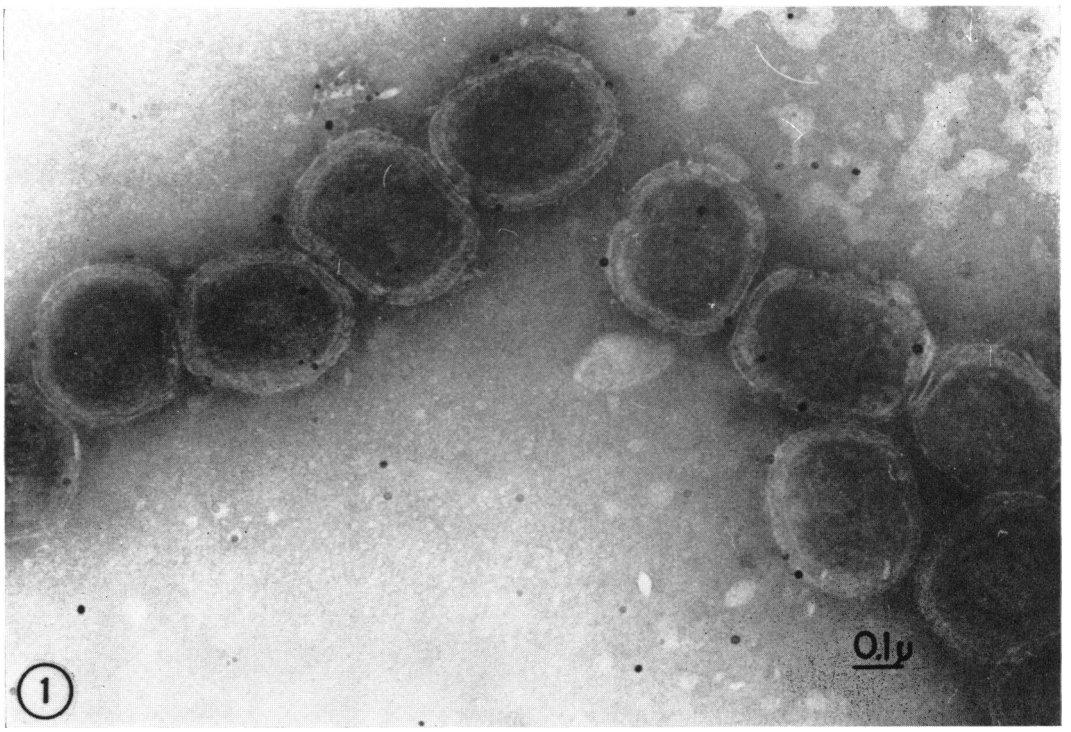

FIG. 1. *Purified preparation of fowlpox virus negatively stained with PTA (pH 7.0) showing C forms.* ×*60,000.*

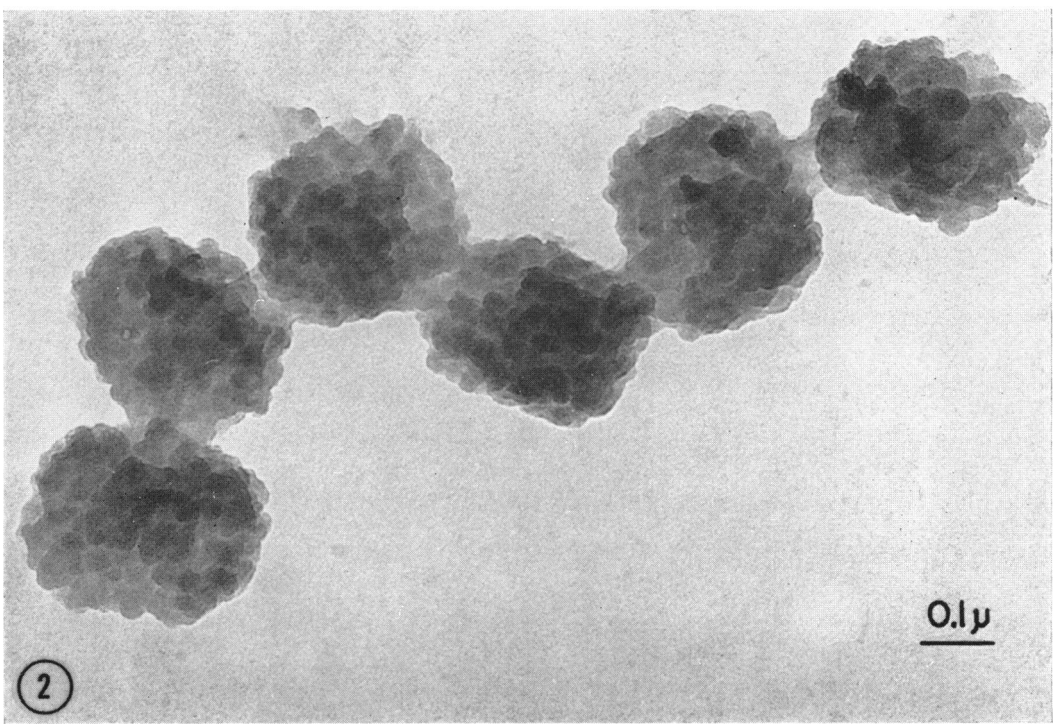

FIG. 2. *Purified preparation of fowlpox virus stained with 0.007% UA and shadowed lightly with platinum at an angle of 60°. C forms exhibit an irregular knobby surface layer. Note adhesion of the particles.* ×*90,000.*

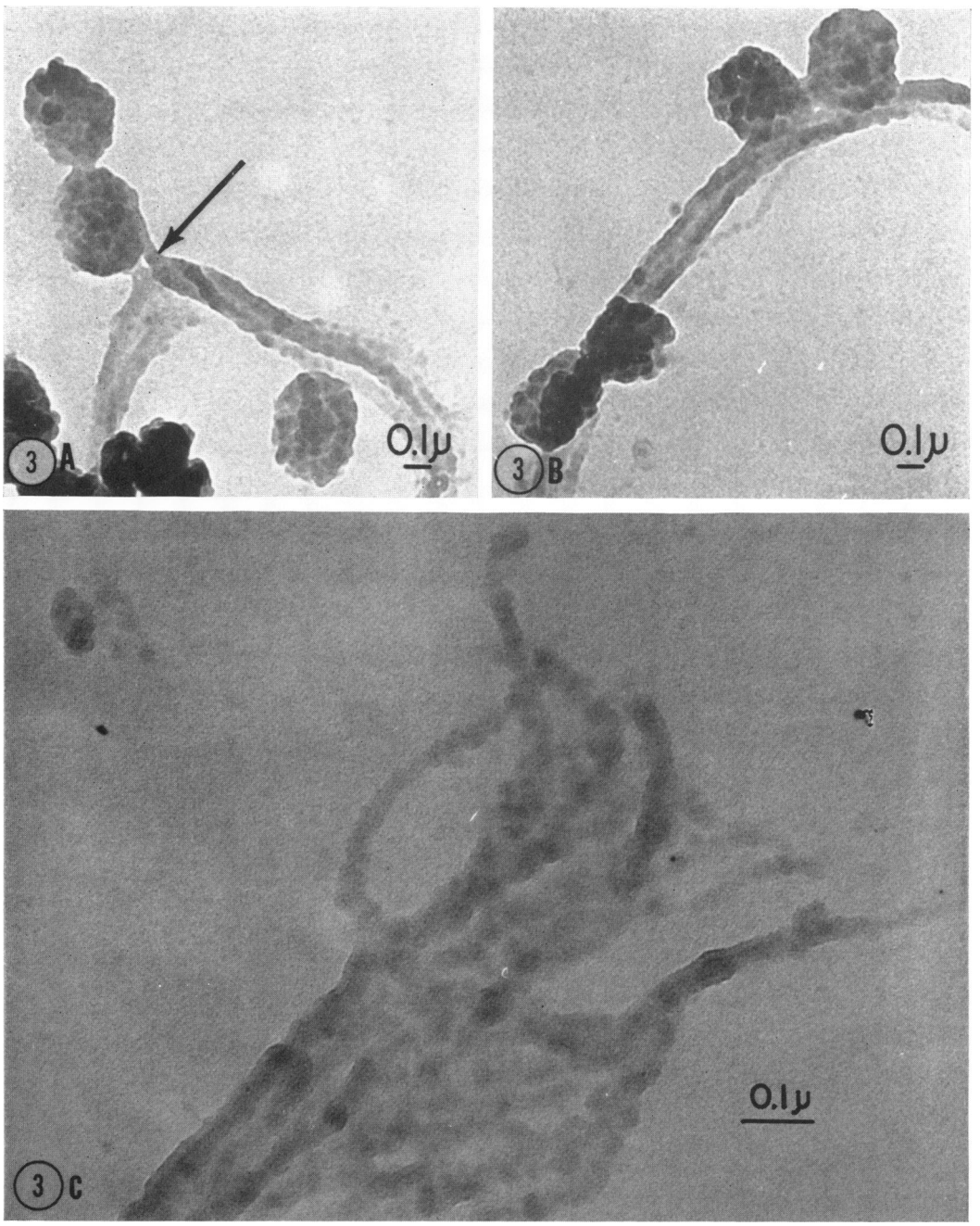

FIG. 3. *Purified preparation of C forms stored for about 1 year at 4 C. Preparations were stained and shadowed as described in Fig. 2. Subunits from the external layer(s) of the particles have apparently aggregated into a "rope-like" configuration. (A) Arrow indicates the site of attachment of a virus to the "rope." (B) Particles are shown intimately adherent to this structure. (C) High magnification of the frayed end of the same coil of subunits. Note dense components within the strands, and compare with Fig. 5. A, ×40,000; B, ×40,000; C, ×100,000.*

obtained from frozen and thawed infected chick embryo monolayer cultures, and frozen and ground infected chick scalp and chorioallantoic membrane. The crude material, suspended in 0.02 M phosphate buffer, was partially clarified by light centrifugation in a clinical centrifuge (800 × *g*), or by gravity sedimentation for several hours. Pseudoreplicas of the resulting supernatant fluid were then prepared. Figure 4 shows the M form of FP particles, with its peripheral arrangement of rodlets or tubular structures. Attempts to further purify the M forms resulted in "conversion" to C forms. This was evident after one preliminary cycle of centrifugation in the Spinco model L ultracentrifuge and resuspension in buffer.

Effect of trypsin on whole virus. Fresh, purified suspensions of C forms were treated with 1% trypsin to elicit unwinding of the outer coat(s) of the virus. Pseudoreplicas of treated virus were stained with 0.5% UA and shadowed with platinum. In Fig. 5, an outer layer appears to be unwinding from the virus particle; other particles are in various stages of disintegration. Three distinct, darkly staining strands are present in the central portion of this outer coat, and one hairlike strand (marked with arrow) extends beyond the free end of the "unpeeling" coat. The knobby appearance (Fig. 2) was not seen after trypsin treatment.

Internal structure studied with negative staining at alkaline pH. Negative stains at neutral *p*H have been described, and PTA stains made at an acid *p*H were unsatisfactory. A study of virus negatively stained at various alkaline *p*H levels was suggested by the work of Peters and Muller (1963) and Peters, Muller, and Buttner (1964), who demonstrated that the nucleoids of vaccinia and milker's nodule virus (paravaccinia) were both composed of a triad of large oblong units.

Pseudoreplicas of FP were stained with 0.5% PTA at *p*H 8.0, 9.0, 10.0, 10.5, and 11.0. Penetration of fowlpox particles by the stain was observed at *p*H 8 and appeared to be optimal at *p*H 9. Values of *p*H higher than 9 caused distortion of the particles. Figure 6A shows a viral particle stained at *p*H 10.5 that is extensively penetrated by PTA and in the process of dissolution with the apparent evulsion of internal material. It is noteworthy that the hollow shell remaining shows only a single membrane layer, and the intact particle (Fig. 6B) exhibits two distinct layers. The thickness of the inner layer appears to be about 200 to 300 A. Occasionally, at *p*H 9.0 (Fig. 7), particles were seen with a faintly visible internal structure, possibly analogous to the internal triad of vaccinia virus seen by Peters and Muller (1963). However, the nucleoid usually appeared as a centrally located, poorly defined mass.

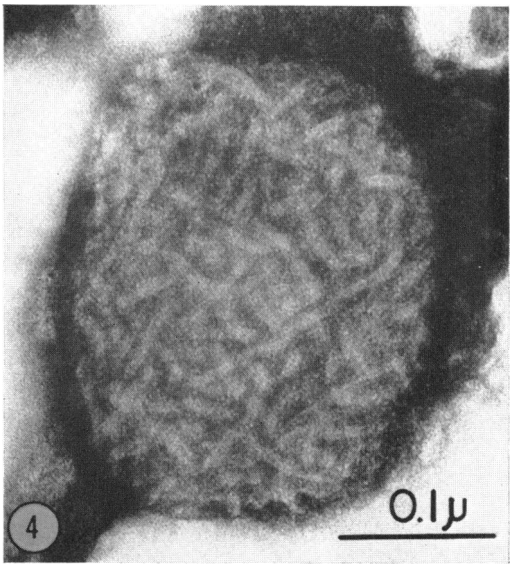

FIG. 4. *Impure preparation of fowlpox virus. Negative stains with PTA (pH 7.0) reveal M forms whose surfaces are covered with rodlets or hollow tubules.* ×*200,000.*

Ultrathin sections. Pellets of virus were embedded in Epon 812, sectioned on a Porter-Blum ultramicrotome, and stained with UA. The appearance of thinly sectioned FP often resembled that of vaccinia virus (Peters, 1962; Dales, 1963) with outer membranes, lateral bodies, and a biconcave-shaped nucleoid. These were practically identical to images of FP in choroiallantoic membrane (Arhelger and Randall, 1964). Figures 8 and 9 are thin sections of FP stained with UA and show a detail of intertwining dense strands in the interior of the particles, possibly analogous to the dense filamentous components in the nucleoids of immature and mature forms of vaccinia virus (Dales, 1963). The nuclear triad, described in thin sections of vaccinia virus by Peters and Muller (1963) and in paravaccinia viruses (Peters et al., 1964; Buttner et al., 1964), was not identified as such in thin sections of fowlpox virus; however, conditions of fixation and staining were different, suggesting the need for further study of the effect of various methods of fixation on viral structures. Enzyme histochemistry (Bernhard and Tournier, 1962) should prove particularly useful in determining the nature of the nucleoid strands (presumably DNA-protein complexes).

Exposure of DNA strands by detergent action.

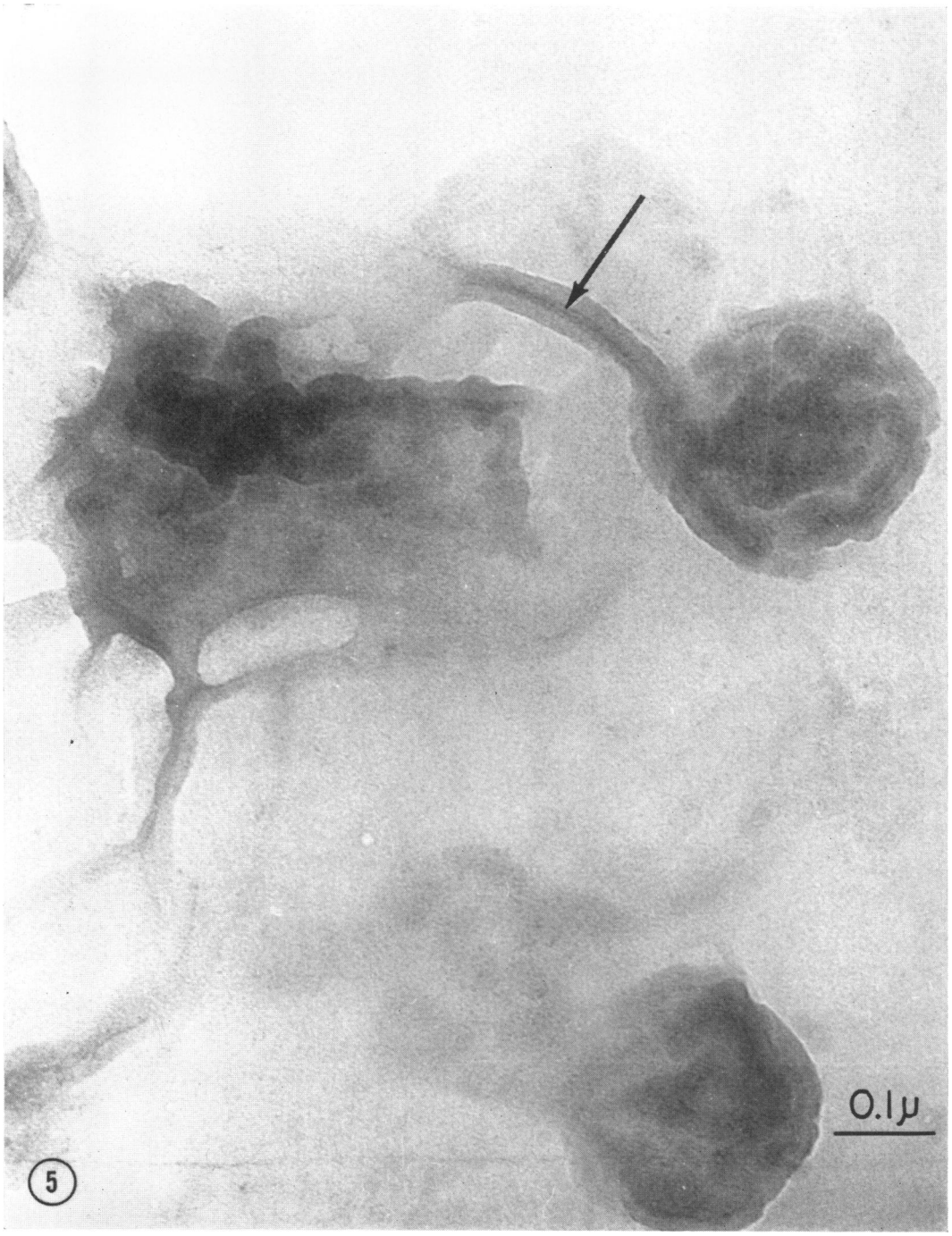

FIG. 5. *Purified preparation of C forms treated with trypsin and stained with UA. Note dense strands within the partially detached virus coat (marked with arrow). Other viral particles are in various stages of disintegration, with fragmentation and loss of structure.* ×*150,000.*

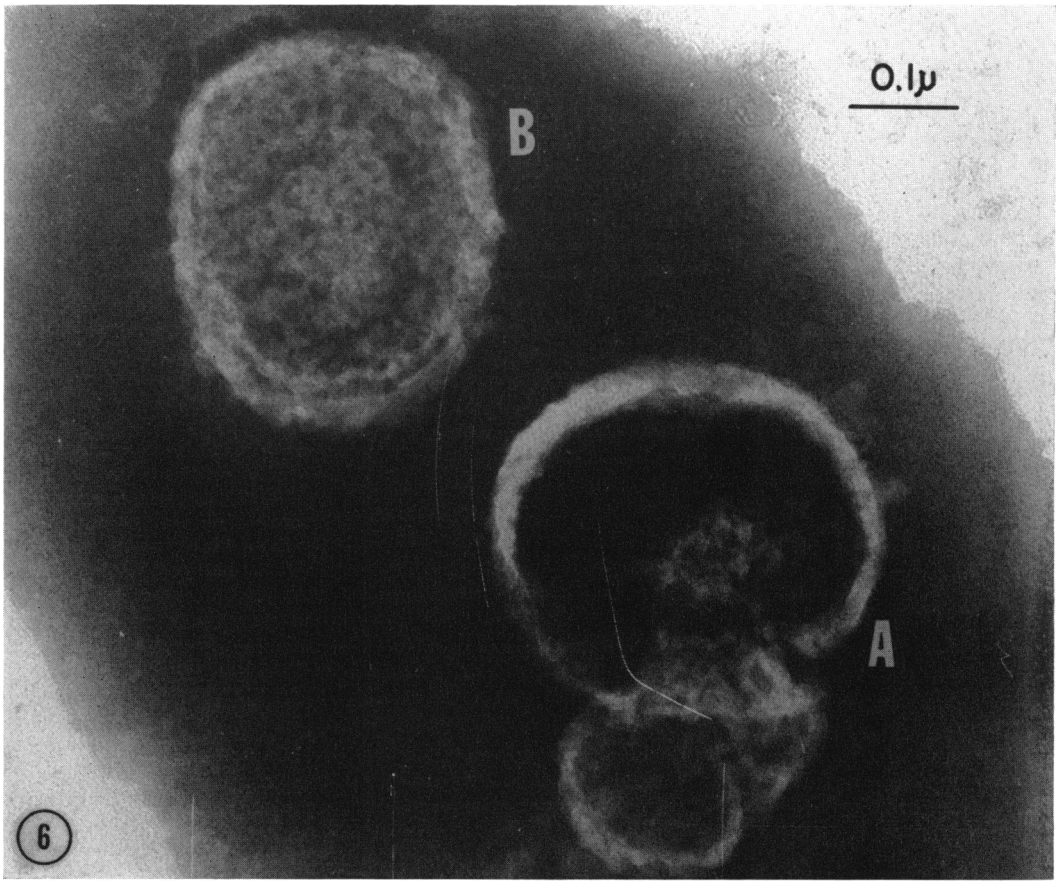

FIG. 6. *Purified virus stained with PTA at pH 10.5. Particle A appears to be sloughing some of its contents. The intact virus (B) exhibits two membrane structures.* ×*150,000.*

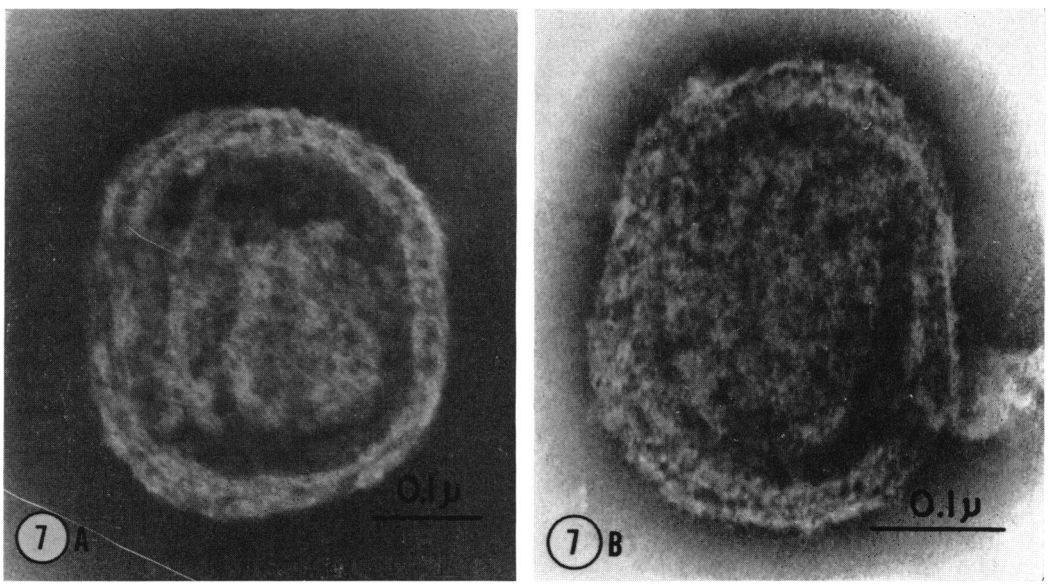

FIG. 7. *Purified virus stained with PTA at pH 9. The penetration of PTA reveals nucleoid structures with coiled or folded appearance.* ×*160,000.*

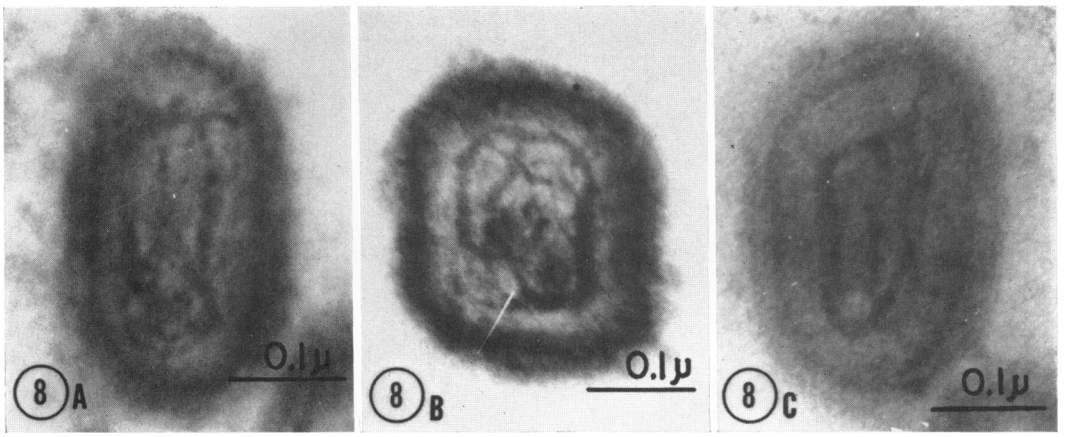

FIG. 8. *Three micrographs of ultrathin sections of fowlpox virus stained with UA. Dense intertwined strands are present in the nucleoid region of each virus particle.* ×*150,000.*

Subviral infectious material (largely DNA) has been extracted from fowlpox virus by use of SLS (Randall, Gafford, and Soehner, 1964). It had been assumed that treatment with SLS caused lysis or dissolution of the virus with liberation of DNA. To visualize, by use of electron microscopy, strands of DNA in the process of extraction from viral particles, the following experiment was devised. Pseudoreplicas of SLS-virus mixtures were prepared and stained with 0.5% UA. Examination of these preparations (Fig. 10) revealed particles with a shape similar to fowlpox virus. The comparatively light staining of these particles attested to the fact that they were altered in some manner, and treated virus particles were somewhat smaller than untreated ones. The darkly stained nucleoids were composed of uranophilic coiled strands which extended for considerable distances beyond the periphery of virtually every particle observed. The strands were frequently seen extending from one particle to another (Fig. 11). Complete nucleoids were not observed outside the particles and, apparently, were not extracted as such.

Effect of nucleases and trypsin on DNA strands. It appeared reasonable to assume that the strands noted above might be DNA or DNA-protein. To test this possibility, SLS-virus mixtures on agar were treated with 100 μg/ml of deoxyribonuclease for 15 min at room temperature, and pseudoreplicas were prepared. The filaments were digested by deoxyribonuclease (Fig. 12); however, the strands were intact after treatment with deoxyribonuclease buffer, trypsin, and heated or unheated ribonuclease. It is noteworthy that treatment with deoxyribonuclease at 37 C for 30 to 60 min resulted in further digestion of the DNA within the virus particles. The network of uranophilic material was obliterated, and most of the particles resembled hollow spheres.

The knobby appearance of the outer layer was not seen in any of the UA-stained SLS-treated preparations. However, the suggestion of very small subunits showing on the surfaces of the extracted viral particles (Fig. 11) prompted the use of neutral PTA. With this procedure, the subunits, approximately 40 A in diameter, were somewhat better defined (Fig. 12). This inner layer may be analogous to the palisaded layer in vaccinia observed by Westwood et al. (1964).

DISCUSSION

It is apparent from these studies that the combination of uranyl acetate staining and platinum shadowing of the C form of fowlpox results in morphological detail clearly different from that shown by PTA staining. It is questionable whether the interpretation of vaccinia C forms as being damaged or empty (Dales, 1962) can be applied to fowlpox virus prepared from inclusions. Fowlpox virus prepared in this laboratory appears to be of good quality, since it is highly infectious, has prominent nucleoids, and contains a fairly constant amount of lipid, protein, and DNA (Randall et al., 1963). Furthermore. the physicochemical properties of extracted DNA indicate that it is highly polymerized (Szybalski et al., 1963). Certainly these particles are not empty. It is probably true, however, that the C forms represent some modification or loss of an external coat, and it is possible that C forms prepared by some method other than that used in this study will not present a knobby irregular surface.

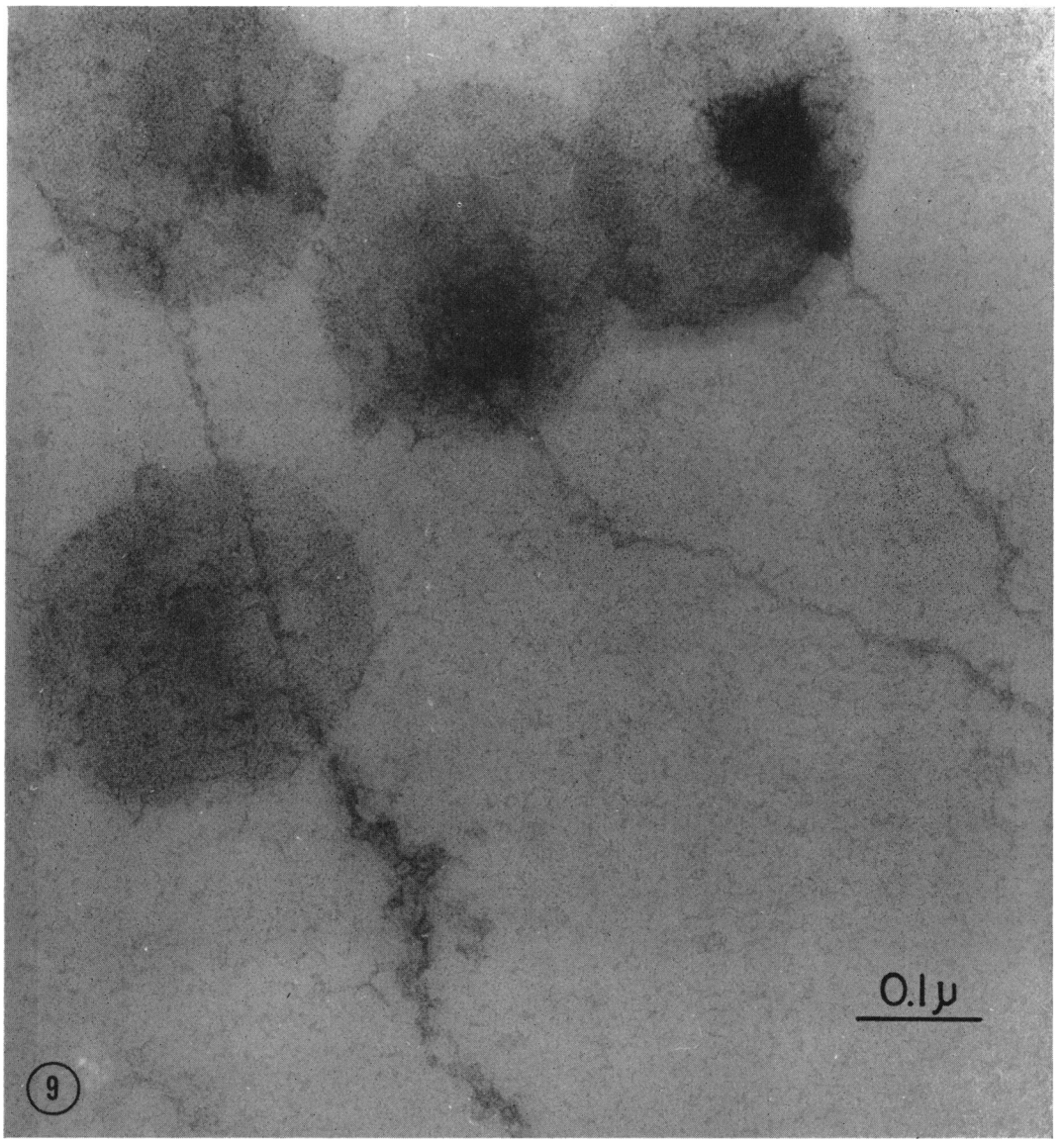

FIG. 9. *Purified fowlpox virus treated with SLS and stained with UA. Note the dark strands (DNA or DNA-protein) arising from nucleoid material and extending beyond the periphery of the viral particles into the supporting film.* ×*170,000.*

Westwood et al. (1964) have investigated the conversion of M to C forms of vaccinia under a variety of conditions. They concluded that the appearance of the M form was due to exclusion of PTA from the interior of the virus, and that penetration resulted in the C form.

The unwinding of the outer coat of the virus into long strands and the partial uncoating with trypsin lead one to consider the possibility that intracellular virus formation may be completed by the winding of strands, or by the linear addition of individual subunits around the core.

It is clear that treatment of the virus with SLS releases some of the DNA without completely destroying the virus structure. Nevertheless, the virus is no longer infectious (Randall and Gafford, *unpublished data*), confirming the work of Bang, Levy, and Gey (1951*a*, *b*).

After the initial effect of SLS, there is no noticeable change in the morphological appear-

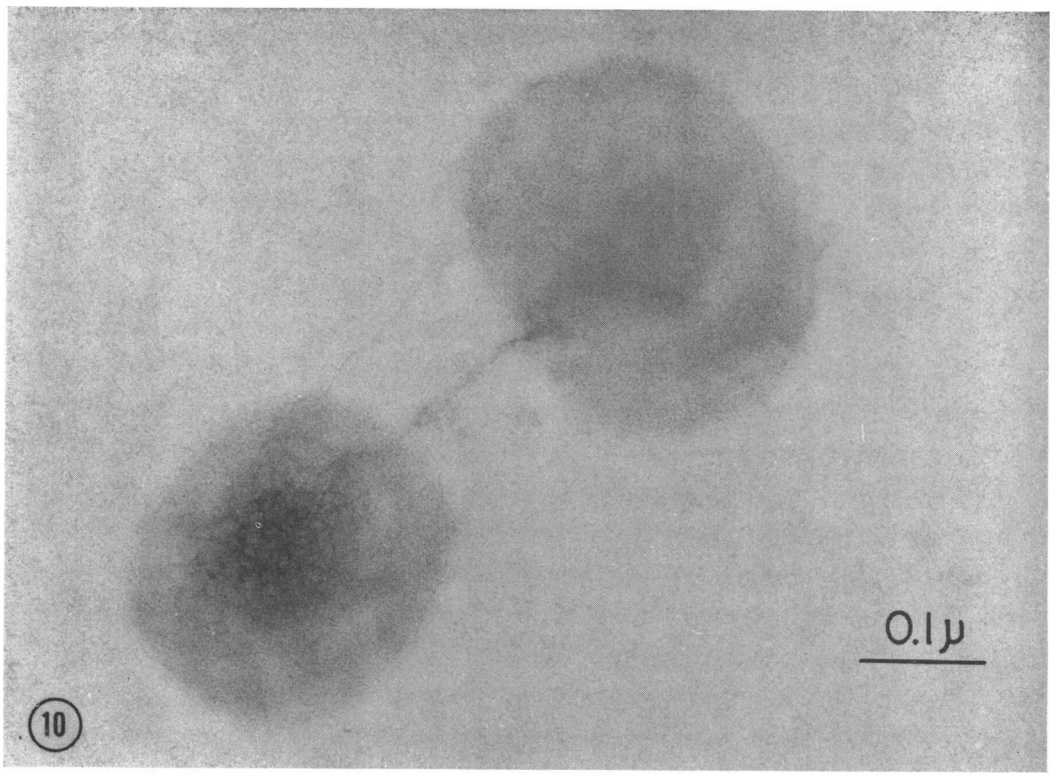

FIG. 10. *Two viral particles apparently connected by DNA strands after SLS treatment. DNA can be seen coiled within the particle on the left. UA stain.* ×*170,000.*

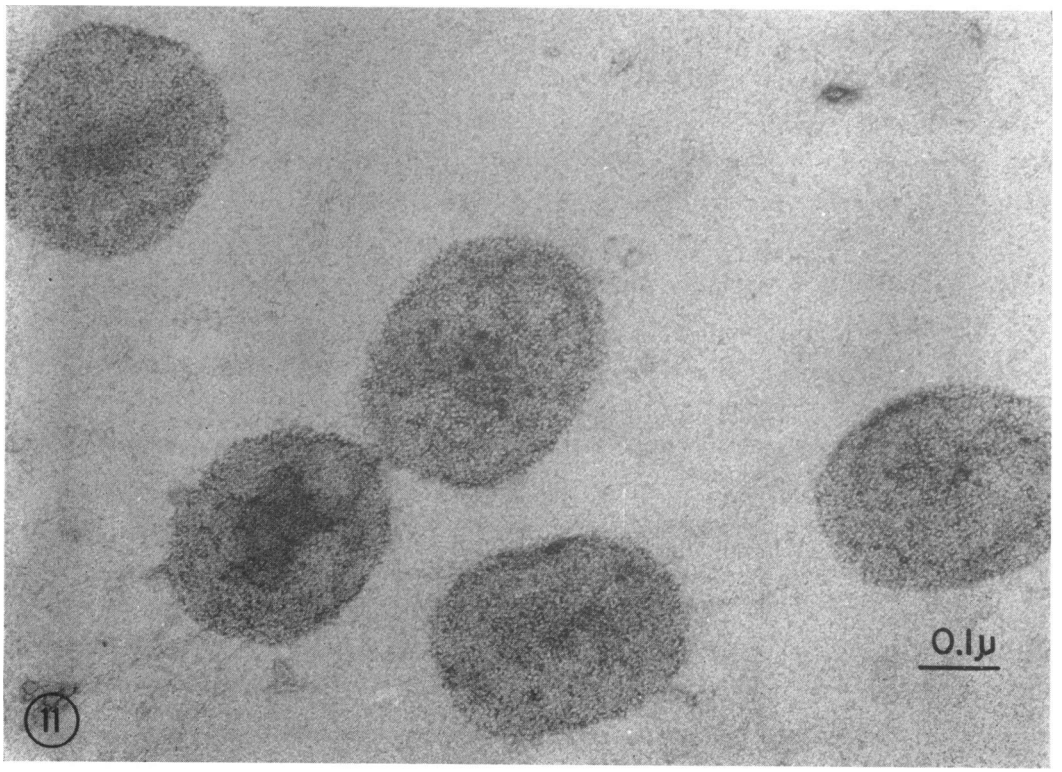

FIG. 11. *DNA strands from SLS-treated particles are absent, or only faint remnants remain, after deoxyribonuclease treatment for 15 min. The nucleoids remain to a varying degree, but the coiled DNA is no longer evident. UA stain.* ×*110,000.*

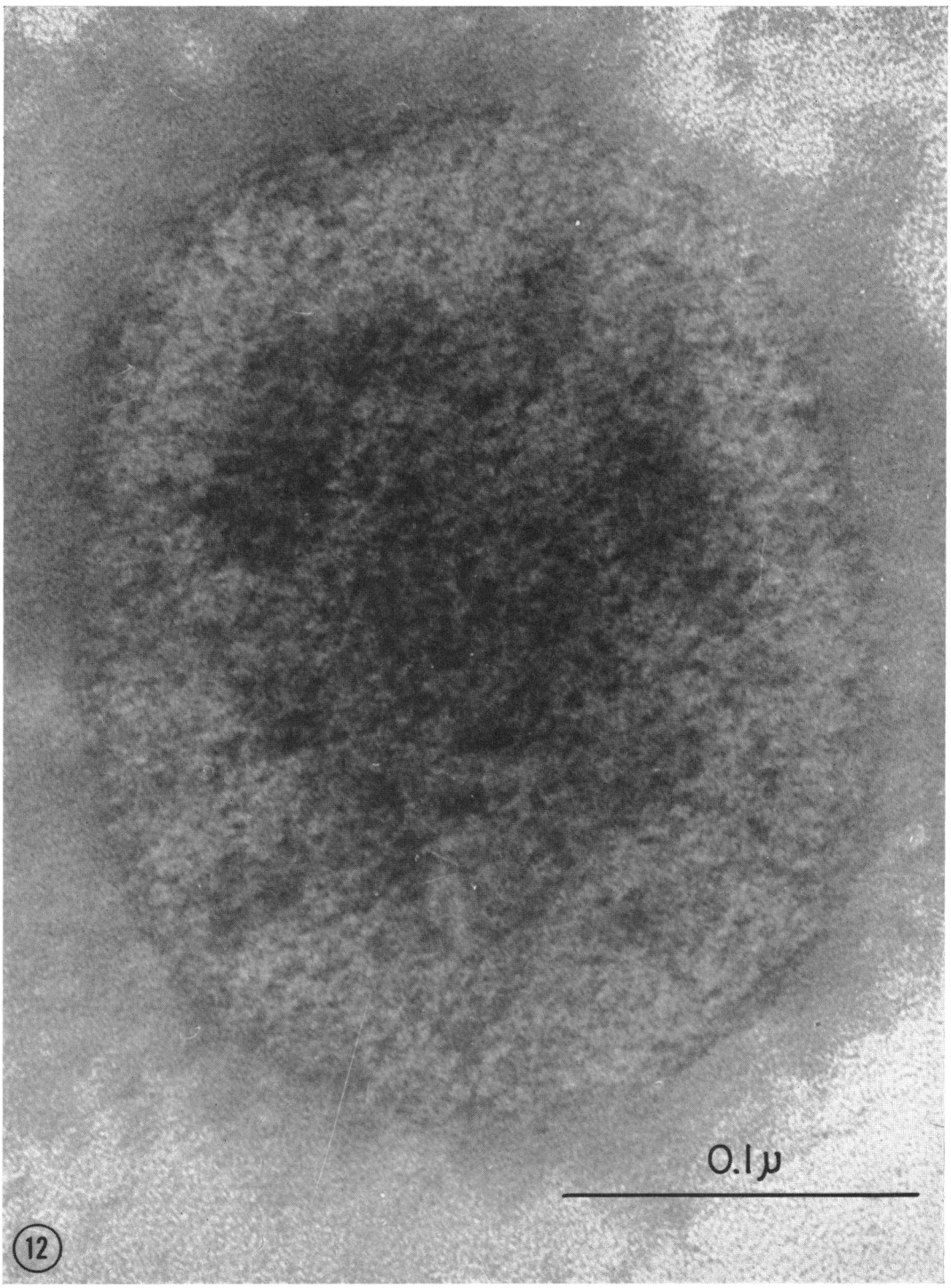

FIG. 12. *Neutral PTA stain of virus previously degraded by SLS and treated with deoxyribonuclease for 15 min. This treatment appears to expose very small subunits.* ×*520,000.*

ance of the virus, even after storage at 4 C in 2% SLS for several weeks. It is not known whether the nucleocapsid (Casper et al., 1962) is normally permeable to DNA and the function of the outer coat(s) is to keep the virus intact, or whether the release of DNA is brought about by exposure of the nucleocapsid to SLS, a known denaturant of proteins (Putnam, 1948). The results of these experiments indicate the need for further studies of the effects on the nucleoid of various agents which remove the outer coat(s) of the virus.

The failure of negative stains to reveal structural details of the FP nucleoid appears to be related to the penetrating ability of neutral PTA solution. Alkaline PTA (*p*H 9) penetrates the virus readily and may outline a complex nucleoid structure. The severe damage resulting from exposure to *p*H values above 9 suggests that findings made with alkaline PTA stains may be artifactual, and that caution should be used in interpreting results.

The most promising means of determining the internal structure of fowlpox is ultrathin sectioning, although the exact interpretation of the dense strands seen in sections in unclear. The evidence indicates that they are nucleoprotein in nature, as the strands are centrally located within the virus, and are strongly uranophilic as is characteristic of DNA. Preliminary experiments with the acetic-alcohol fixative of Peters and Muller (1963) have yielded little additional information in this regard. The effects of various methods of fixation on nucleoid configuration are now under investigation.

The present studies have demonstrated the complexity of fowlpox virus and the necessity for the use of diverse methods in examining it. Experiments are under way to determine, by electron microscopy and ultrastructural cytochemistry, the nature of the various components of fowlpox virus and the inclusion body.

ACKNOWLEDGMENTS

We thank Stephen H. Randall and Janice Van Zandt for excellent technical assistance.

This investigation was supported by Public Health Service training grant AI-69 and research grant AI-02031 from the National Institute of Allergy and Infectious Diseases, grant AM-07496 from the National Institute of Arthritis and Metabolic Diseases, and grant GMA-07115 from the Division of General Medical Sciences.

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