MORPHOLOGICAL STRUCTURE OF THE VIRUS OF VACCINIA

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Plates 20 and 21

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The morphology of the agents responsible for variola and vaccinia has been the subject of extensive discussion for a number of decades. Of the many forms described by various workers only two are now considered significant; these are the cytoplasmic inclusions of Guarnieri and the minute particles or elementary bodies of Paschen (1). The identification of the elementary body with the infective unit of vaccinia has been established as a result of careful experimentation in several laboratories over a period of years (2–6). Moreover, the idea that the cytoplasmic inclusion represents a colony of elementary bodies has been held by certain workers (7). Elementary bodies of vaccinia are relatively large (236–252 mμ in diameter as estimated from ultracentrifugation data (8)) and this accounts for the frequency with which they have been examined in the stained state by ordinary microscopy, as well as by means of ultraviolet light in unstained preparations (9), and more recently by the aid of the electron microscope (10). In spite of this work evidence of internal structure in the elementary body has not previously been recorded. The purpose of this report is to present a series of electron micrographs which show that the virus particles of vaccinia possess definite morphological structure.

Materials and Methods

Virus.—Suspensions of washed elementary bodies of vaccinia were prepared by a method (11) based on the technique of Craigie (4). For certain of the lots of virus, however, the machine recently described by Pickels (12) was used instead of the Swedish angle centrifuge. Satisfactory sedimentation has been obtained with this new apparatus by spinning the virus suspension at 12,400 R.P.M. for 20 minutes in lusteroid tubes having an internal diameter of 11 mm. Sedimented virus was resuspended with the aid of a rubber plunger which had been shaped to conform closely to the side and bottom of the tube. This modification of the original technique of Craigie (4, 11) saves time, and the final virus preparations are of the same degree of purity (6) since their infective unit-elementary body ratios compare favorably with those of lots prepared in the angle centrifuge.

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Preparation of Specimens for Electron Microscopy.—The general technique of electron microscopy has been recently described (13); hence, only the details of the procedure which are of importance in the study of vaccinia need be mentioned. One drop of a stock suspension of washed virus was placed on a thin collodion membrane which was attached to a fine mesh wire screen for support. A few seconds later the drop was removed as completely as possible with a fine capillary pipette. In this way relatively few virus particles were brought into contact with the membrane; those which became attached were rarely lost during subsequent manipulation because of the adhesive nature of the surface of the elementary body. The surface of the membrane was then thoroughly washed by submersing it repeatedly in a solution of physiological saline and subsequently in distilled water. After excess fluid had been removed by means of a capillary pipette, the preparation was rapidly dried in the air and placed in the RCA electron microscope (13). Preliminary observations indicated that the effect of various agents on the structure of the virus could best be studied by applying the test materials directly to films of active elementary bodies which had been prepared as described. Following treatment the films were again washed in saline solution and in water.

EXPERIMENTAL

Elementary bodies of vaccinia when viewed by electron microscopy present a high degree of regularity of external outline and of internal form. The particles are almost rectangular in shape and usually possess five circumscribed areas which are more dense than the surrounding substance and hence appear darker in the electron micrograph, as shown in Fig. 1*a*. The central area of condensation in the elementary body is slightly larger than the others which are spread around it; their general arrangement suggests that of the five spots on dice (Fig. 1*b*). Elementary bodies which are joined by a narrow bridge of material of lighter density than the bodies themselves are not infrequently encountered (Fig. 1*a*). In one instance a break in the collodion film with a subsequent folding back of the newly formed edge occurred during exposure to the electron beam. This permitted a side view of the particles which in the new position appeared to have flat tops and bottoms (Fig. 4) similar to the side walls when viewed from above (Fig. 1*a*). Thus, the general shape of the virus particles seems to resemble a brick. Electron micrographs of preparations of elementary bodies which had been rendered non-infectious by heating at 56°C. for 1 hour were indistinguishable from those of active virus particles. Similarly, films of elementary bodies prepared in the usual way and then completely dehydrated in a vacuum jar over phosphorus pentoxide showed virus particles with the regular internal structure. Finally, specimens with elementary bodies which had been frozen while on the collodion membrane and then dried from the frozen state contained virus particles with areas of condensation similar to those shown in Fig. 1*a*.

Alkaline treatment of elementary bodies liberates a nucleoprotein antigen

(14) from the particles without completely dissolving them. The structures which remain after extraction stain less deeply with silver, are less brilliant in dark field illumination, and sediment less readily in the centrifuge than do active elementary bodies (14). Therefore, it seemed desirable to observe by electron microscopy the various stages of dissolution of elementary bodies caused by alkali. Partially and completely extracted virus particles are illustrated in Figs. 2 and 3. The virus is noticeably affected by treatment with N/50 NaOH for ½ minute at room temperature (Fig. 2). Certain of the bodies are swollen with rounded outlines and fuzzy edges; their substance is less dense than normal and their areas of condensation stand out prominently. Extraction with N/10 NaOH for 10 minutes lakes the virus particles almost completely, and even the condensed areas have practically disappeared (Fig. 3). Certain of the ghosts formed in this manner have ruptured; some have wedge-shaped gaps in their surfaces and others show substance streaming from them.

The morphological evidence obtained by electron microscopy does not permit one to designate the limiting structure which separates the virus from the surrounding medium as a semipermeable membrane. All that can be said is that the surfaces of the bodies under examination appear to be different from the main internal constituents. This is borne out by the occurrence of bridges of material joining aggregated elementary bodies (Fig. 1*a*) and by the streaming out of internal substance from one point on the surface (Fig. 3). The nature of the limiting membrane remains to be determined.

The high degree of purity of the final preparations of virus is clearly demonstrated in the electron micrographs. The paucity of extraneous material in those portions of the field between elementary bodies in Fig. 1*a* is immediately apparent. Crude suspensions of dermal pulp which are rich in elementary bodies contain much non-viral material. Films prepared from supernatant fluid obtained from such suspensions after the virus had been sedimented contain innumerable tiny particles of different sizes and shapes (Fig. 5). The supernatant fluid was applied to the film and subsequently washed in a manner identical with that used for suspensions of virus, yet no structures which simulate elementary bodies are seen (Fig. 5). While part of the amorphous material shown in this figure might consist of the soluble LS-antigen of vaccinia, most of it probably represents small bits of cellular debris; in any case, it is discarded in the process of purifying the virus. The granular material present in the background of Figs. 2 and 3 is probably nucleoprotein which was extracted from the elementary bodies by alkali and which was not completely removed from the film by subsequent washing. It will be recalled that this nucleoprotein is relatively insoluble in aqueous solutions having a pH near 7.0 (14).

Soluble proteins are easily washed from the collodion films by rinsing in

saline solution and then distilled water. Fig. 6 illustrates the findings in a film of elementary bodies prepared in the usual way and then treated with a drop of antivaccinal rabbit serum and subsequently washed. Little if any of the antiserum remains on the membrane for the background is essentially clean. Some of the antibody has apparently remained attached to the virus particles, however, for they are more dense than usual since the electron beam failed to penetrate sufficiently to bring out internal structures such as are seen in Fig. 1*a*. Normal rabbit serum employed in similar experiments is removed from the virus by the subsequent washing, as evidenced by the elementary bodies so treated having sharp outlines and recognizable areas of condensation. It must be emphasized that inadequate washing of virus particles immediately prior to examination in the electron microscope will give results like those illustrated in Fig. 6. This is true even when untreated stock suspensions of purified virus are employed. Inasmuch as a few ghosts are encountered in all suspensions studied, there apparently is sufficient constant destruction of elementary bodies to provide protein which is adsorbed on the surface of the remaining elementary bodies, thus rendering them more opaque than those illustrated in Fig. 1*a*.

The results of an additional control experiment are illustrated in Fig. 7. A suspension of triturated normal rabbit kidney tissue was subjected to the same procedure of differential centrifugation employed for vaccine virus and films were prepared in the usual way from the final suspension of particles. None of the material represented in Fig. 7 resembles elementary bodies closely and most of it is strikingly different from the virus. The large spherical masses are interesting but their nature is not apparent; they may represent certain of the particles observed by ordinary microscopy in preparations from normal cells (15).

DISCUSSION

Former observations made in the course of our studies of the virus of vaccinia have led us to believe that this agent might be regarded as a complex entity which perhaps resembles certain bacteria more closely than it does the crystalline plant viruses. Indeed, the virus of vaccinia may differ principally from members of the bacterial species in its size and fastidious growth requirements; only living cells from certain animal species are capable of serving as adequate culture media for the virus and this may be because the elementary bodies lack some enzyme systems essential for independent growth and metabolism. The basis for the idea that the virus of vaccinia resembles certain bacteria has been provided by several types of experimental data. For example, elementary bodies apparently respond to osmotic influences (16), they have certain chemical and biologically active constituents in common with living cells (11, 17), and they are antigenically complex (14, 18). The observations recorded in this paper indicate that vaccine virus has a

morphological structure which in many respects approaches that of bacteria (10, 19) and differs from that of the plant viruses (20).

SUMMARY

The pictorial data obtained by means of the electron microscope indicate a remarkable regularity in the morphology of the elementary body of vaccinia. The virus particles apparently have internal structure and some sort of limiting membrane.

BIBLIOGRAPHY

1. Blaxall, F. R., Smallpox, in A system of bacteriology in relation to medicine, London, His Majesty's Stationery Office, 1930, **7,** 99.
2. Eagles, G. H., and Ledingham, J. C. G., *Lancet,* 1932, **1,** 823.
3. Nauck, E. G., and Paschen, E., *Centr. Bakt.,* **1** *Abt., Orig.,* 1932, **124,** 91.
4. Craigie, J., *Brit. J. Exp. Path.,* 1932, **13,** 259.
5. (*a*) Parker, R. F., *J. Exp. Med.,* 1938, **67,** 725. (*b*) Parker, R. F., Bronson, L. H., and Green, R. H., *J. Exp. Med.,* 1941, **74,** 263.
6. Smadel, J. E., Rivers, T. M., and Pickels, E. G., *J. Exp. Med.,* 1939, **70,** 379.
7. Bland, J. O. W., and Robinow, C. F., *J. Path. and Bact.,* 1939, **48,** 381.
8. Pickels, E. G., and Smadel, J. E., *J. Exp. Med.,* 1938, **68,** 583.
9. Barnard, J. E., *Brit. J. Exp. Path.,* 1935, **16,** 129.
10. von Borries, B., Ruska, E., and Ruska, H., *Klin. Woch.,* 1938, **17,** 921.
11. Hoagland, C. L., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.,* 1940, **71,** 737.
12. Pickels, E. G., *Rev. Scient. Instr.,* 1942, **13,** 101.
13. (*a*) Marton, L., *J. Bact.,* 1941, **41,** 397. (*b*) Zworykin, V. K., Hillier, J., and Vance, A. W., *Elec. Eng.,* 1941, **60,** Trans. 157. (*c*) Anderson, T. F., The study of colloids in the electron microscope, in Kraemer, E. O., Advances in colloid science, New York, Interscience Publishers, Inc., 1942.
14. (*a*) Smadel, J. E., Lavin, G. I., and Dubos, R. J., *J. Exp. Med.,* 1940, **71,** 373. (*b*) Smadel, J. E., Rivers, T. M., and Hoagland, C. L., *Arch. Path.,* 1942, in press.
15. (*a*) Claude, A., *Tr. New York Acad. Sc.,* 1942, **4,** 79. (*b*) Rous, P., and Robertson, O. H., *J. Exp. Med.,* 1917, **25,** 651.
16. Smadel, J. E., Pickels, E. G., and Shedlovsky, T., *J. Exp. Med.,* 1938, **68,** 607.
17. (*a*) Hoagland, C. L., Lavin, G. I., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.,* 1940, **72,** 139. (*b*) Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., *Proc. Soc. Exp. Biol. and Med.,* 1940, **45,** 669. (*c*) Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.,* 1941, **74,** 69. (*d*) Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.,* 1941, **74,** 133.
18. (*a*) Smadel, J. E., and Rivers, T. M., *J. Exp. Med.,* 1942, **75,** 151. (*b*) Shedlovsky, T., and Smadel, J. E., *J. Exp. Med.,* 1942, **75,** 165.
19. (*a*) Mudd, S., and Lackman, D. B., *J. Bact.,* 1941, **41,** 415. (*b*) Morton, H. E., and Anderson, T. F., *Proc. Soc. Exp. Biol. and Med.,* 1941, **46,** 272.
20. (*a*) Kausche, G. A., Pfankuch, E., andRuska, H., *Naturwissenschaften,* 1939, **27,** 292. (*b*) Anderson, T. F., and Stanley, W. M., *J. Biol. Chem.,* 1941, **139,** 339.

EXPLANATION OF PLATES

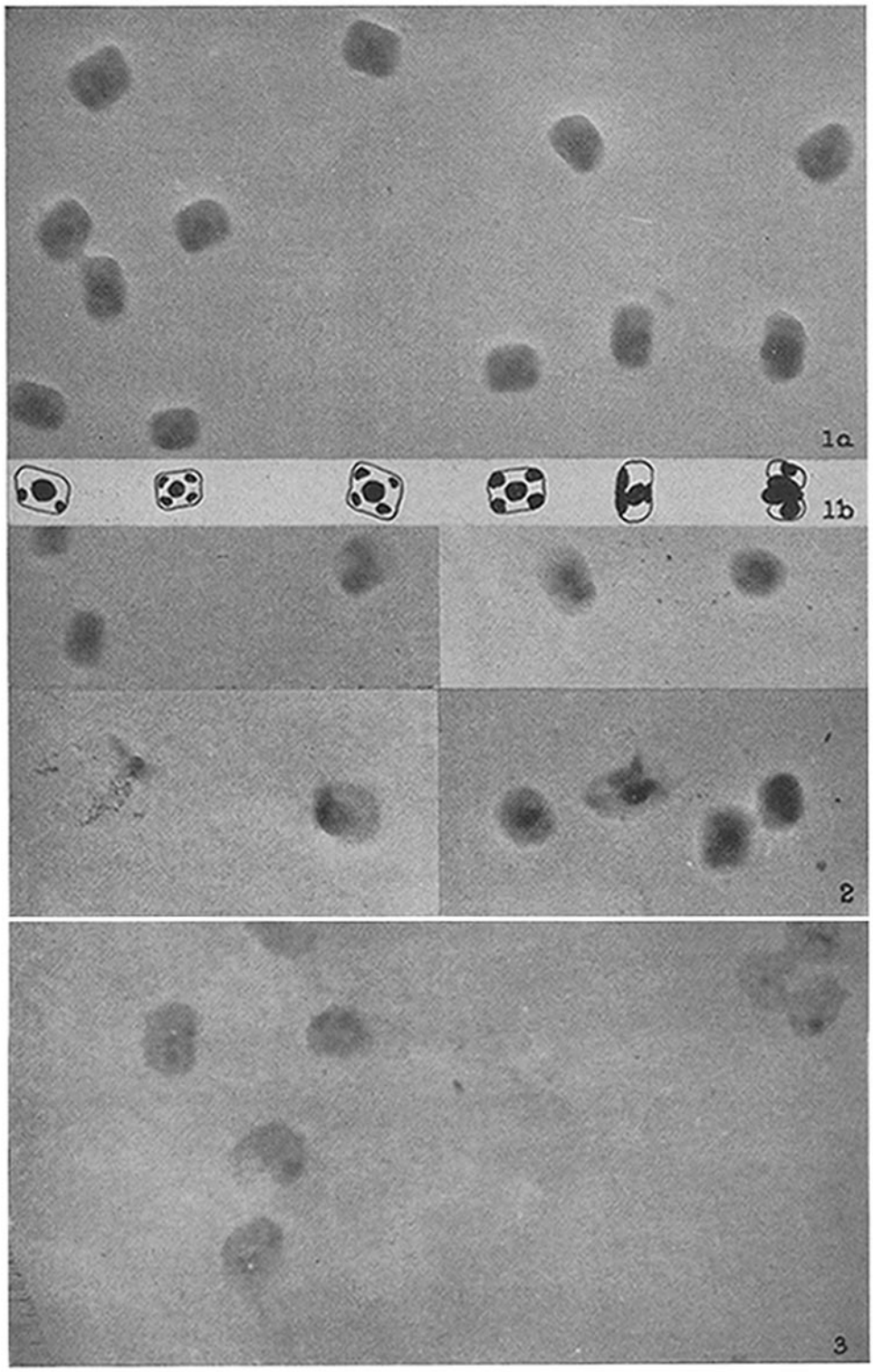
PLATE 20

FIG. 1 *a*. Reproduction of an electron micrograph of a purified preparation of elementary bodies of vaccinia. Rectangular shaped virus particles containing dark circumscribed areas are seen. Little extraneous material is present. Magnification 7100 × 4.

FIG. 1 *b*. A schematic representation of certain of the virus particles shown in Fig. 1 *a*; each drawing is placed beneath the body it typifies.

FIG. 2. Combined portions of several electron micrographs illustrate the effects of N/50 NaOH on Paschen bodies. Some virus particles appear almost normal; *i.e.*, they are rectangular with sharp outlines and internal areas of condensation. Others are swollen and rounded with fuzzy outlines; the substance of these bodies is pale and the darker areas of condensation stand out prominently. The granular debris probably represents nucleoprotein extracted from the virus particles. Magnification 7100 × 4.

FIG. 3. Elementary bodies extracted with N/10 NaOH. The virus particles have been laked by the alkali; they are swollen and pale and the areas of condensation have almost disappeared. Certain bodies show wedge-shaped breaks in their surfaces. A stream of internal substance appears to have flowed from the elementary body at the bottom of the figure. Magnification 7100 × 4.



(Green Anderson, and Smadel: Morphology of vaccine virus)

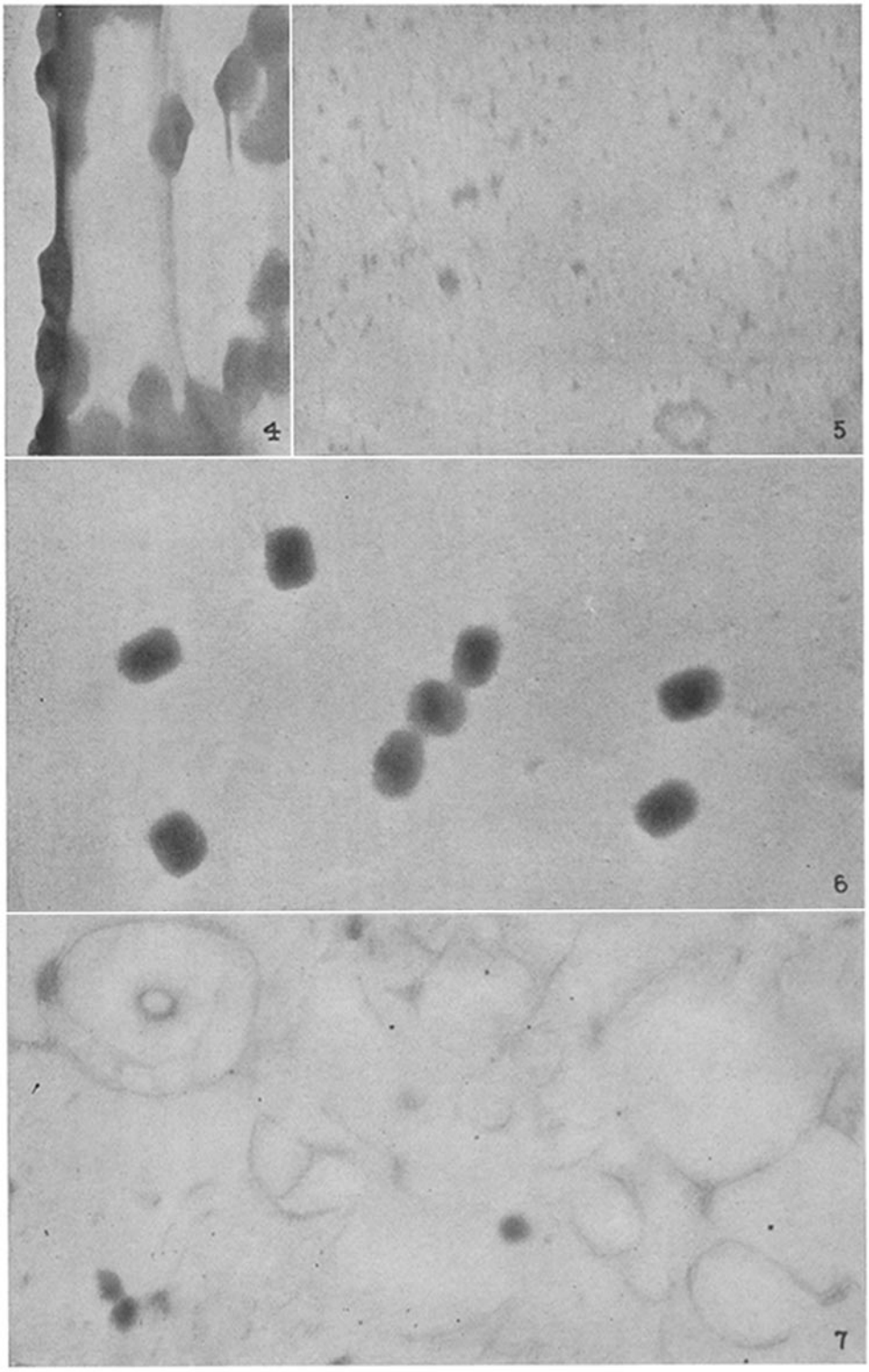
PLATE 21

FIG. 4. The collodion membrane has broken and folded back, revealing some of the virus particles in profile. The bodies seen at the left of the figure appear to have essentially flat tops and bottoms. Hence, the shape of the elementary bodies seems to resemble a brick. Magnification 7100 × 4.

FIG. 5. A suspension of crude vaccine dermal pulp from which the virus particles have been removed by centrifugation. No structures which resemble elementary bodies are seen. Magnification 7100 × 4.

FIG. 6. A film of elementary bodies such as those illustrated in Fig. 1 *a* was prepared and then treated with a drop of antivaccinal serum. This was washed off with saline solution and water. Evidence of internal structure in the virus particles is not convincing and the outlines of the bodies are not sharp. A similar effect is produced by the non-specific adsorption of other types of protein on the surface of the elementary bodies (see text). Magnification 7100 × 4.

FIG. 7. Normal kidney cell particles were obtained from a suspension of rabbit kidney tissue by the same procedure of differential centrifugation employed for vaccine virus. Bizarre structures are present in the illustration but none simulate the elementary bodies seen in Fig. 1 *a*. Magnification 7100 × 4.



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