ELECTRON MICROSCOPE STUDY OF RED CELL MEMBRANES AFTER EXPERIMENTAL INFECTION WITH THE VIRUS OF FOOT-AND-MOUTH DISEASE*

BY B. EPSTEIN, V.D.,‡ N. M. FONSECA,§ M.D., AND E. DE ROBERTIS, M.D.

(*From Departamento de Ultraestructura Celular, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay*)

PLATES 19 TO 21

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The phenomenon of red cell agglutination by virus has been used with considerable success for the differential adsorption of viruses (1, 2) and for their study with the electron microscope (3–5). The intact erythrocyte is too thick for ordinary transmission electron microscopy but red cells lysed osmotically or by other means yield ghosts which are partially transparent to the electron beam (6). The observation of the fine structure of the membrane and adsorbed virus particles can be improved considerably with the shadow-casting technique.

With these methods Heinmets (3) could demonstrate the adsorption of active influenza B virus and A and B virus inactivated with formalin on chick and human erythrocytes. Dawson and Elford (4, 5) using laked fowl red cells improved the technique considerably and were able to study the adsorption of influenza A and B virus, fowl plague virus, Newcastle disease virus, and mumps virus. A quantitative analysis of the size of virus particles and of the number of virus particles adsorbed per unit area in relation to virus concentration and other conditions was carried out by these authors.

Recently Michelsen and Bachrach (7) found that agglutination of rat erythrocytes at pH 6 is produced by guinea pig lymph infected with the virus of foot-and-mouth disease. This fact indicates that the virus can be adsorbed differentially upon the erythrocytes. The study of this adsorption process with the electron microscope seemed to us rather difficult in view of the small dimension attributed to this virus (8).

The older literature had some indications that the problem of virus–cell host relationship in foot-and-mouth disease could be attacked by the electron microscope from other angles. In fact, in addition to the marked epitheliotropic characteristics of the virus, localization in the blood and other tissues could

* Work supported by a grant from The Rockefeller Foundation.

‡ Fellow from the Instituto de Biología Animal de Montevideo.

§ Fellow from the Instituto de Ginecología, University of Brazil, Rio de Janeiro.

be demonstrated. From 24 to 48 hours after exposure to the infection, generalization of the disease takes place and the blood becomes infective.

Valée and Carré (9) indicated the presence of the virus of foot-and-mouth disease associated with the red cells during the period of generalization and postulated the possibility of hemovaccination by using infective blood, while Graub, Zschokke, and Saxer (10), impressed by the presence of the virus in red cells, prepared a vaccine with virulent blood treated with crystal violet.

These facts indicated the possibility that by studying the red cell membranes of infected animals, at different periods of the disease, changes related to the presence of virus particles could be observed with the electron microscope. Furthermore, tests of infectivity of the red cells could be made by inoculating into susceptible animals.

Material and Techniques

The Vallée O type strain of foot-and-mouth disease virus obtained from the Instituto de Higiene Experimental de Montevideo[1] was used in these experiments. This strain of bovine origin had been adapted to the guinea pig by regular passages for 2 years. The virus suspension was prepared by triturating, in sterile sand, vesicle walls with equal parts of buffer phosphate of pH 7.6. The suspension was centrifuged and the supernatant was inoculated after filtration through a Seitz filter.

A total of twenty-five guinea pigs was inoculated. Inoculation of 0.5 ml. was performed intradermally in one of the posterior pads of the guinea pig by making multiple confluent tracks with a No. 25 needle. Generalization of the disease with secondary vesicles in the other pads and tongue was regularly obtained in 48 hours. At this time 5 ml. of blood was drawn by heart puncture and coagulation was prevented with heparin. The red blood cells were repeatedly washed in physiological saline solution and then injected in the proportion of 0.5 ml. intradermally and 0.5 ml. subcutaneously. Generalized infection was obtained within 72 hours indicating the presence of the virus in association with the red blood cells. The lymph obtained from this experiment served as pool of infective material for the series of experiments described below. Blood smears were prepared from different specimens and at times after inoculation ranging from 24 to 92 hours. Signs of disease such as generalization of the vesicles, amount of lymph, and fever were recorded in each experiment and correlated with the electron microscope results. Parallel experiments of virus transmission from washed red blood cells at different times after infection were performed. Positive inoculations were obtained at 42 hours coinciding with the generalization of vesicles and temperatures ranging from 39.5° to 40.3° C. Negative results were obtained after 92 hours of inoculation when general symptoms and temperature were declining.

The blood smears were hemolyzed with distilled water and the red cell membranes transferred upon standard nickel grids by a replica technique with parlodion film. Control of the results was made by direct observation with the electron microscope, but final study was generally done after shadowing with palladium at grazing angles of 9–11°. An R.C.A. type EMU 2C microscope was used and microphotographs were generally taken at about × 4,000 to × 6,000.

[1] We would like to express our gratitude to Dr. E. Hormaeche, Director of the Instituto de Higiene Experimental, for the use of this virus strain and for his critical reading of the manuscript.

OBSERVATIONS

Red Cell Membranes of Normal Control Guinea Pigs.—Five normal guinea pigs served as control animals. In each case several blood smears were taken and a large number of grids prepared from different regions of the smear. About 10 grids were examined for each control or experimental animal and the maximum number of squares (about 30) was observed with the electron microscope. Since for each square of the grid there were about 50 red blood cells, a total of several thousand cells was examined in each case.

Observations on normal red cell membranes were extended to other mammalian species and also to avian erythrocytes studied with the same technique. In several hundred electron micrographs of red blood cell membranes from normal, human, mouse, bovine, pig, chicken, and pigeon cells, the fine structure of the ghost showed very slight differences as compared with normal guinea pig erythrocyte (Fig. 1). Such a comparative description is beyond the scope of this work but we want to point out that in no case could inclusions similar to those found in the experimental animals be observed.

Fig. 1 shows one of the fifty electron micrographs taken from normal red cell membranes of the guinea pig. They appear as almost perfectly flat membranes 10 mμ thick. Shadow-casting permits the observation of very fine details of structure on the surface and shows a clean background. In such a membrane any material in the form of particles or group of particles larger than 30 or 40 mμ will stand out as regions of higher electron density or protrusions on the surface.

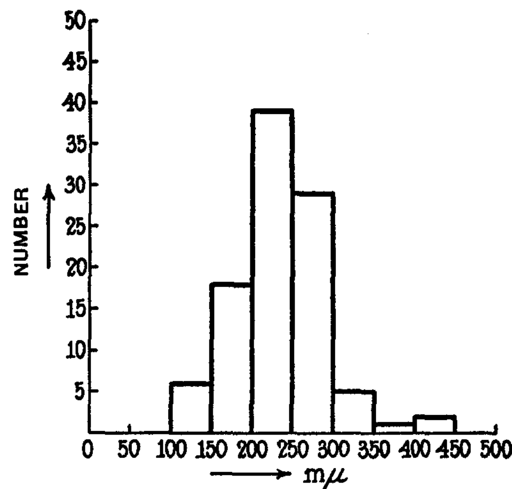
Red Cell Membranes of Guinea Pigs Infected with the Virus of Foot-and-Mouth Disease.—Eight of the inoculated guinea pigs were utilized for the electron microscope observations. The study of the blood smears was done in the same way as in the controls. The following description is based not only on a total of 105 electron micrographs, but on the observation of several thousand cells in each case.

Fig. 2 shows a typical electron micrograph of the red cell membranes of a guinea pig, *24 hours* after inoculation with the virus. All four membranes pictured show the presence of rounded masses with higher electron density, distributed at random on the surface of the erythrocytes. The size of these masses ranges from 100 to 500 mμ and their number is of the order of 20 or more per cell. The limit of these dense masses is not very sharp in most of the cases and the shadow-casting demonstrates that they protrude only slightly above the surface. The observation of the electron micrographs and the direct observation with the electron microscope permit one to conclude that practically all the erythrocytes exhibit the alteration illustrated in Fig. 2.

In Figs. 3 and 4 are shown the most conspicuous changes observed *42 hours* after inoculation with the virus. Fig. 3 is a direct positive print of a specimen very lightly shadowed with palladium so that this treatment interferes very

little with actual differences in electron density of the membrane and of the masses. These round patches are denser and more clearly defined than at 24 hours after inoculation and they show a tendency to be distributed near the edge of the membrane in a ring-shaped disposition. The number of these masses is about 25 to 30 per cell; in some cases it is difficult to count them exactly because they join laterally. The diameter of most of the masses ranges from 200 to 500 mμ, but a few smaller ones could also be found.

Fig. 4 is a negative enlarged print of Fig. 3. On this print the masses appear to be constituted of clusters of smaller round entities. Arrows on the figure



TEXT FIG. 1. Distribution curve of measurements of diameter (in millimicrons) of one hundred inclusions of red cell membranes. Guinea pig inoculated 72 hours earlier with the virus of foot-and-mouth disease.

indicate the regions in which these smaller masses are best defined. Although exact measurements are difficult owing to the superposition of the fine structure of the membrane, because of their greater density and packing it is possible to detect particles ranging from 20 to 70 mμ in diameter.

An appraisal of all the electron micrographs taken at this period of the disease in addition to the direct observation of thousands of cells again leads to the conclusion that practically all red cell membranes show the changes illustrated in Figs. 3 and 4.

At this time of the disease, red blood cells repeatedly washed in saline proved to be infective after inoculation in other guinea pigs.

Seventy-two hours after inoculation, round dense masses can be seen which are distributed at random or show some tendency to be disposed in lines (Fig. 5). Analysis of the shadows projected by the masses suggests that they protrude

above the surface (Figs. 5 and 6). The number of masses varies from one membrane to another: 20 to 40 masses per cell are common and in one electron micrograph the unusual number of 90 covered almost the entire surface of the membrane. Measurement of 100 masses taken from several electron micrographs of the same specimen gave a mean diameter of 246 mμ with a range from 116 to 437 mμ. Text fig. 1 shows the distribution curve of these measurements indicating that 86 per cent of the total lies between 150 and 300 mμ. Although some of these masses reach the limit of resolution of the optical microscope, it was not possible to detect them by using this instrument.

The compactness of most of these masses makes it more difficult to determine whether there are smaller masses within. However in some of them this appears to be a possibility. The observation of all the electron micrographs and the direct observation made at this stage with the electron microscope lead, as in the earlier stages, to the conclusion that almost all cell membranes show changes similar to those illustrated in Figs. 5 and 6. This fact is even more striking when one finds that 20 hours later, *92 hours* after inoculation, these masses have disappeared and the red cell membranes have a normal appearance. Inoculation of washed erythrocytes into normal guinea pigs was not infective at this time.

DISCUSSION AND SUMMARY

Guinea pigs were inoculated with the Vallée O type strain of the virus of foot-and-mouth disease. 42 to 48 hours after inoculation red blood cells washed in saline were found to be infective for normal guinea pigs suggesting that erythrocytes carry virus entities at the height of generalization of the disease. This infection is no longer obtained at 92 hours after inoculation when general symptoms of the disease decline. A replica-transfer technique was developed with which hemolyzed blood smears could be observed under the electron microscope.

By direct examination and also shadow-casting with palladium, normal red cell membranes show only a very fine structure on the surface. Rounded masses of high electron density appear in the ghosts between 24 and 72 hours after inoculation with virus. The number and particularly the density of these masses tend to increase from 24 to 42 and 72 hours. In some cases they are disposed preferentially in single lines forming ring figures; in other cases they are distributed at random. At 42 hours after inoculation it was possible to detect dense particles of 20 to 70 mμ within the masses. Measurements of 100 masses at 72 hours gave results ranging from 116 to 437 mμ with a mean diameter of 246 mμ. Results based on 105 electron micrographs and on thousands of cells demonstrate that practically all red cell membranes contained dense masses 24 to 72 hours after inoculation. 92 hours after inoculation, coinciding with the disappearance of infectivity of the erythrocytes, the masses could no longer be seen and the membranes looked entirely normal.

Although the facts reported here may be suggestive of a relationship between the dense masses within the red cell membranes and the presence of virus entities in washed erythrocytes, no definite interpretation of these findings is postulated at present.

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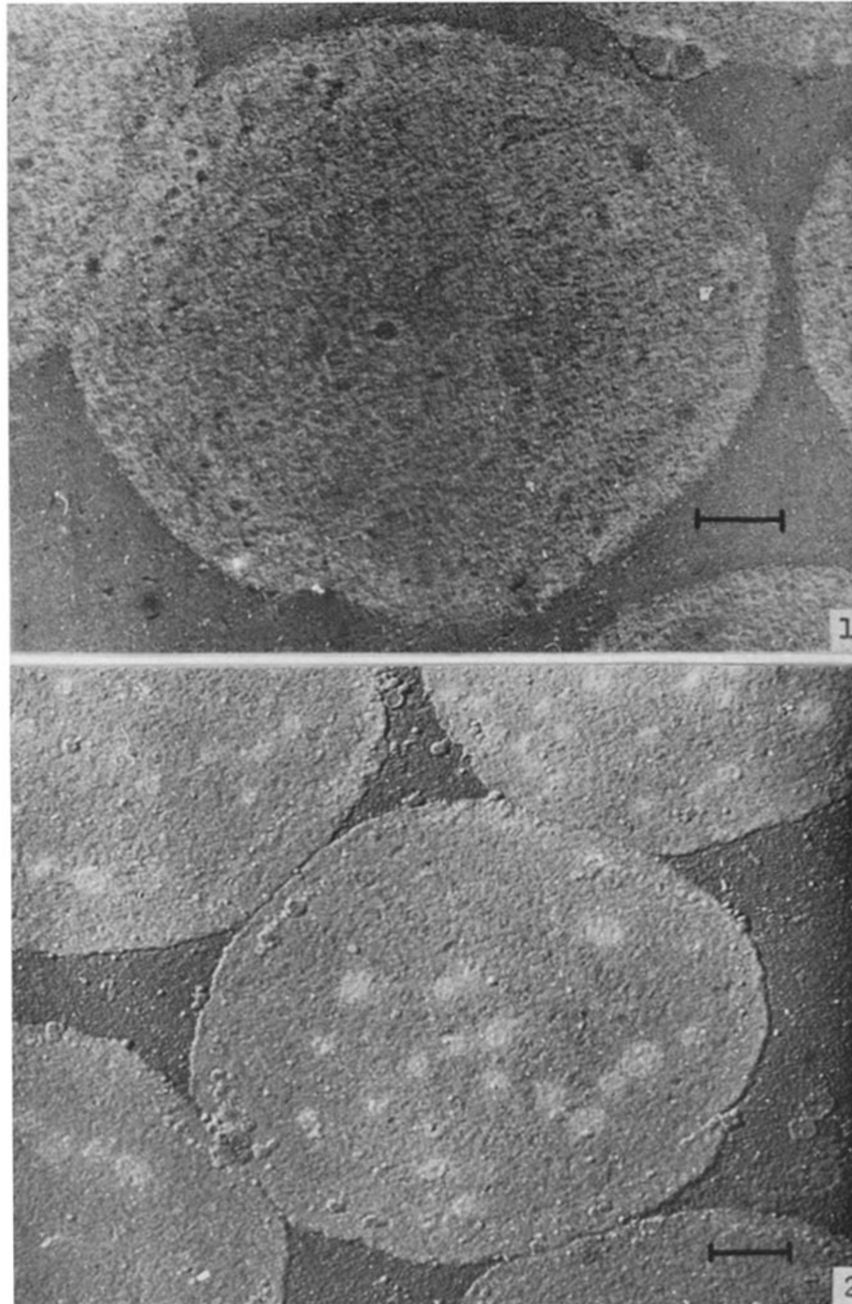
EXPLANATION OF PLATES

All preparations shadow-cast with palladium at an angle of 10°.

PLATE 19

FIG. 1. Red cell membranes from a normal control guinea pig. × 10,800.

FIG. 2. Red cell membranes from guinea pig 27, 24 hours after inoculation with the virus of foot-and-mouth disease, Vallée O type strain. Rounded masses of higher electron density can be observed within the membranes. × 10,600.

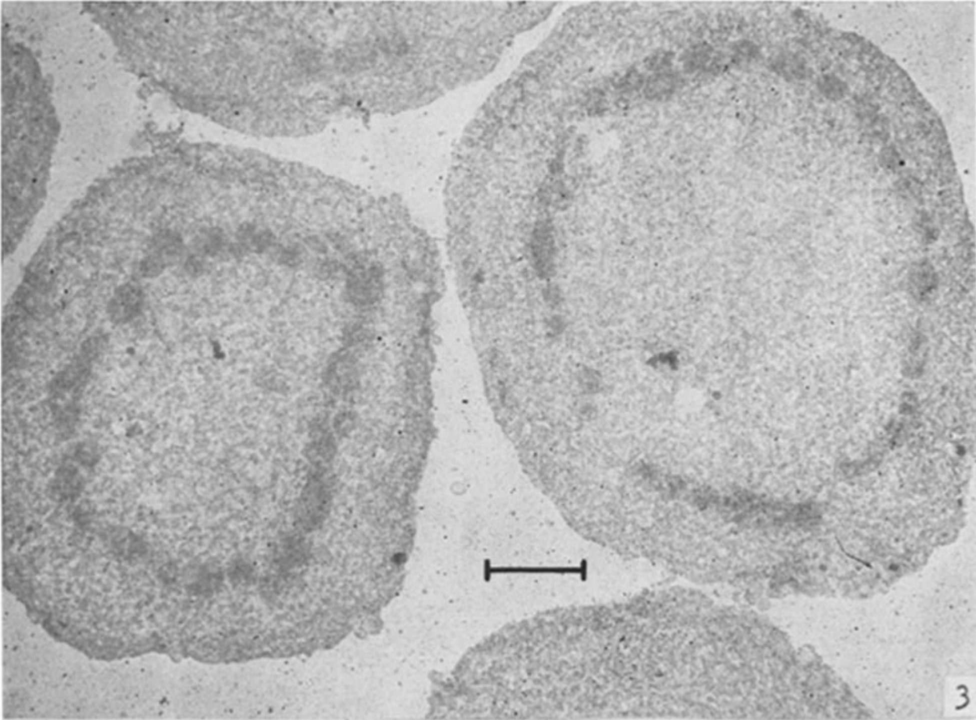


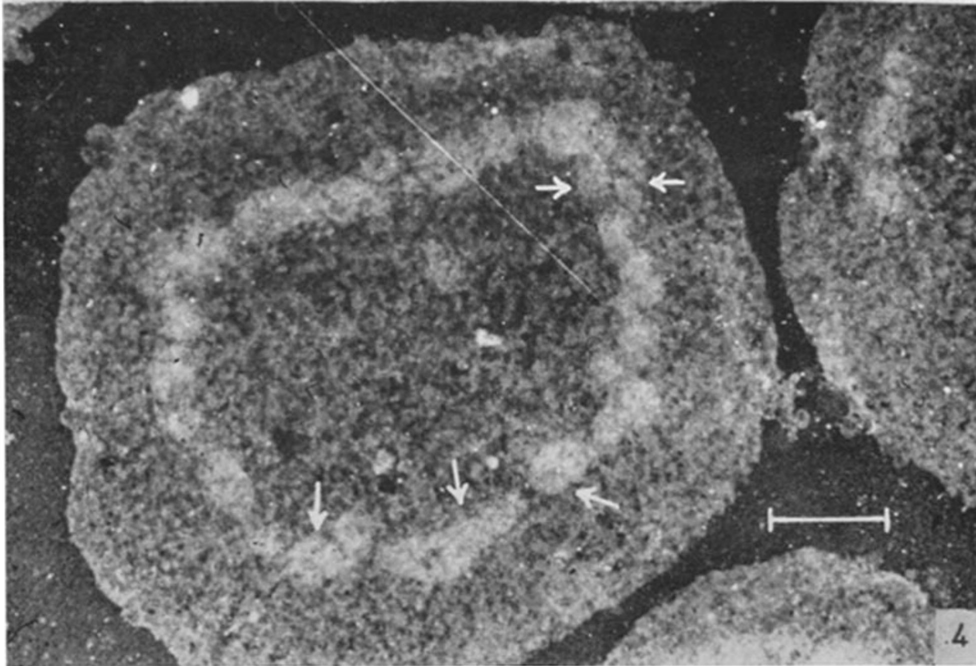
(Epstein *et al.*: Electron microscope study of red cell membranes)

PLATE 20

FIG. 3. Red cell membranes from the same guinea pig as in Fig. 2, 42 hours after inoculation. Positive image. Rounded dense masses mainly distributed in a peripheral ring. × 13,000.

FIG. 4. The same specimen as in Fig. 3. Negative image. Arrows indicate masses in which smaller masses can be best defined. Smallest particles are of the order of 20 mμ. × 16,300.



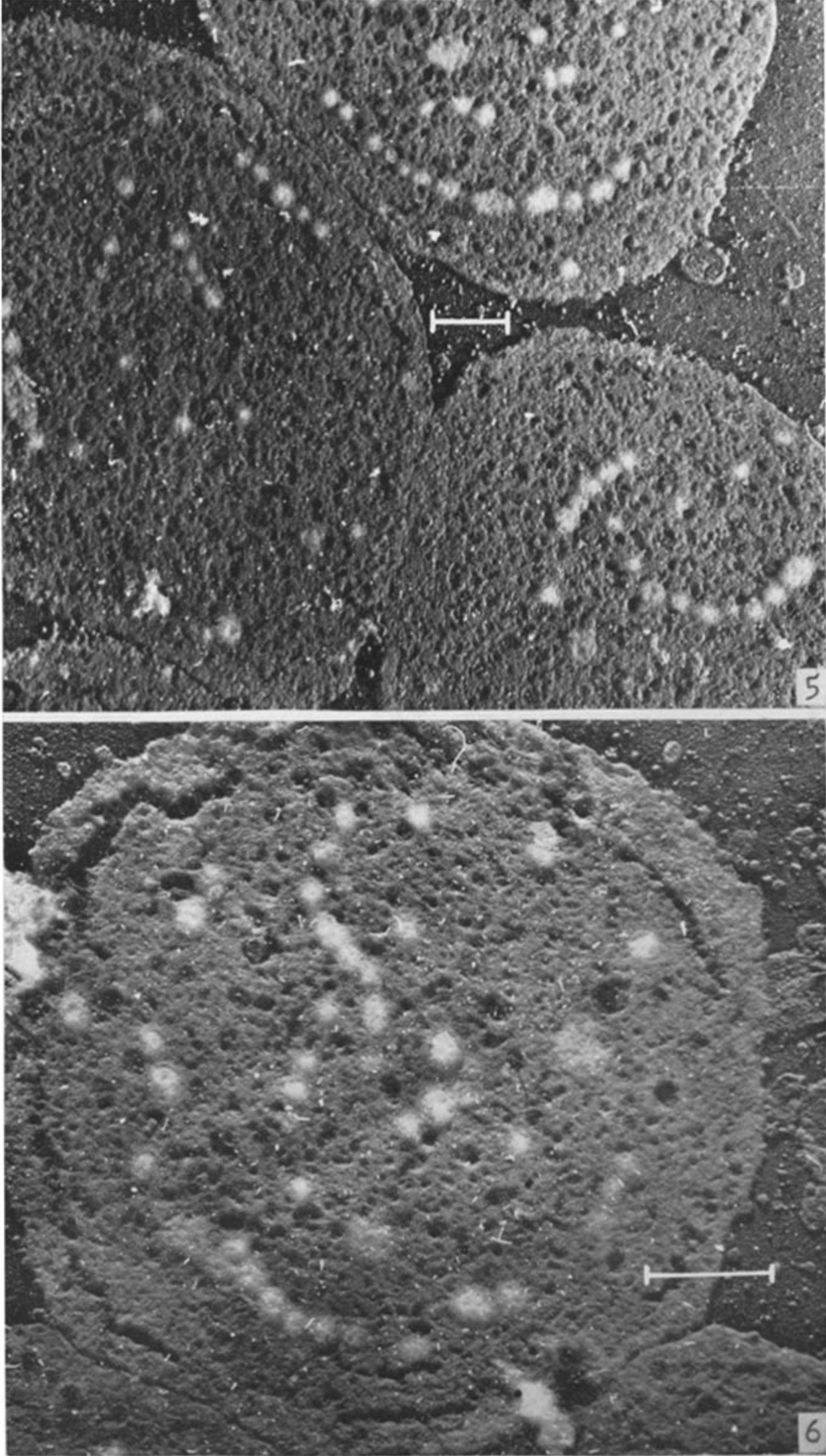


(Epstein *et al.*: Electron microscope study of red cell membranes)

PLATE 21

FIG. 5. Red cell membranes from guinea pig 26, 72 hours after inoculation. Round masses protrude over the surface. They are mainly disposed in single lines. $\times$ 10,500.

FIG. 6. Red cell membrane from the same animal as in Fig. 5. Some of the inclusions show a particulate constitution. $\times$ 17,000.



(Epstein *et al.*: Electron microscope study of red cell membranes)