A MICROSCOPICAL STUDY OF THE REPRODUCTION OF PSITTACOSIS VIRUS.

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SOON after Bedson, Western and Simpson (1930) first demonstrated that psittacosis is caused by a filterable virus, a number of workers described the intracytoplasmic inclusions and " plaques " that are so characteristic of the tissues of animals subjected to this infection (Bedson and Western, 1930 ; Coles, 1930 ; Levinthal, 1930 ; Lillie, 1930).

Bedson and Bland (1932 ; 1934) made detailed morphological studies of the virus as it is seen in stained and fixed preparations of the spleens of infected mice. Bland and Canti (1935) recorded the changes occurring in tissue cultures of infected chick embryo cells by means of motion photo-micrography under dark ground illumination. From these observations it was concluded that psittacosis virus undergoes a complex developmental cycle in which the various changes are linked both in time and morphological sequence.

There is little or no evidence to suggest that this developmental cycle is characterised by an eclipse phase analogous to that postulated for the bacterial viruses. Indeed the results of a recent re-investigation of this question by Bedson and Gostling (1954) based on the microscopy of stained smears and on serological tests for the various forms of the virus together with animal infectivity experiments, give strong support to the opinion that the virus reproduces by a process of binary fission.

The simple hypothesis of binary fission does not however explain the part played in the reproductive process by the " plaques " of the earlier workers. The work of Bland and Canti indicates that these inclusions are in fact colonies of virus embedded in a matrix and enclosed, at least for a time, by a limiting membrane. The limitations of the resolving power of the optical microscope and the disadvantages inherent in dark ground illumination studies precluded the accurate measurement of the range of particle size by these workers and denied to them the observation of the fine details of the intracellular changes. It was therefore decided to examine again the reproductive cycle of psittacosis virus using the greater resolving power of the electron microscope and the improved definition of the structural detail of unstained cells made available by phase contrast microscopy.

MATERIALS AND METHODS.

Strain of virus.—The M.O.H. 154 strain, obtained through the kindness of Professor S. P. Bedson, was used throughout. This strain was adapted to the yolk-sac of the chick embryo and had been passaged in this situation 70 times in the author's laboratory before

this investigation began. Its virulence was such that over 90 per cent of the embryos were killed regularly by 0·1 ml. of a 1/10 dilution of infected yolk sac emulsion 72 hr. after inoculation by the method of Bedson, Barwell, King and Bishop (1949).

The strain was also adapted to the mouse by intraperitoneal inoculation and after 15 serial passages 0·5 ml. of a 5 per cent suspension of infected spleen killed the animals in 48–60 hr.

Strain of mice.—Albino mice from the same stock, 4–8 weeks old, were used throughout.

General plan.

At timed intervals photographic records were made of the changing morphology of the virus during its reproduction. The results of a series of observations, recorded by phase contrast photomicrography, on the cells of the spleen of the infected mouse, were compared with a series of electron micrographs of the various forms of the virus obtained at intervals from the yolk-sacs of infected eggs.

Microscopical examinations.

Optical microscopy.—Impression smears were stained by Bedson's modification of Castaneda's method after fixation with 0·1 N-HCl, by Machiavello's stain, and by Giemsa after alcohol fixation.

Phase contrast microscopy.—Wet preparations of fresh unfixed splenic cells were made from infected mice and from controls which had been inoculated with sterile nutrient broth. To prepare the cell suspensions used in the wet films the spleen of a mouse was removed immediately after the animal's death and was washed in three changes of buffered saline; after fine mincing it was suspended in 1·0 ml. of 5 per cent horse serum in physiological saline and agitated by repeated suction in a wide-bored Pasteur pipette. After allowing the coarse fragments to settle, the supernatant which was rich in detached cells was used to make wet films. Duplicate dry films were prepared for staining at the same time.

Electron microscopy.—Sufficient amounts of the younger forms of the virus could not be obtained from the spleens of infected mice. Pooled yolk-sacs of infected eggs were therefore used as the source of the virus for this part of the work which was controlled by the use of impression smears stained by Castaneda's method.

Three pools, each of at least eight infected yolk-sacs, were collected as follows. The first pool was obtained from infected eggs killed by refrigeration 12 or 18 hr. before the expected death of the embryos; only those yolk sacs in which the large form of the virus predominated in stained impression smears were included. Approximately 20 per cent of the eggs inoculated fulfilled this requirement. The second pool was prepared from eggs which it was anticipated would have died within 6 hr. and in which the movements of the embryo were very sluggish when examined by trans-illumination. The third pool was composed of the yolk-sacs of embryos recently killed by the infection and in which there was a satisfactory content of elementary bodies.

Control preparations were made from the pooled yolk-sacs of uninoculated eggs from each batch used for virus cultivation.

Purification and concentration.

Yolk-sacs were rinsed once in buffered saline and were then broken up by gentle shaking with glass beads. By this method coarse particles of yolk and tissue débris were not fragmented and they could be removed easily by centrifugation. This suspension was then clarified by centrifuging for 5 min. at 1000 r.p.m. The supernatant was then removed and spun in an angle centrifuge for 3 hr. at 4500 r.p.m. This cycle of differential centrifugation was repeated twice and the final deposit was re-suspended in buffered saline. A satisfactory product was only faintly turbid and when a film was stained by Castaneda's stain showed a reasonable virus content.

A drop of the final suspension was placed on a glass slide and fixed for 5 min. in osmic acid vapour. A loopful was then transferred to a collodion membrane on an electron microscope grid and dried over calcium chloride. Dissolved salts were removed by rinsing the grid in distilled water and after drying once more the preparation was shadowed with gold palladium alloy at an angle of 15°. The preparations were examined in an E.M.3 Metropolitan Vickers electron microscope using an accelerating voltage of 75 kV.

RESULTS.

Although Bedson and his colleagues have studied extensively the morphology of psittacosis virus as it is seen in stained impression smears taken from the spleens of infected mice, there is no record of the sequence of events as it occurs in the living cells of this animal. For this reason, and to confirm that the strain of virus used for electron microscopical work reproduced in the classical manner, the morphology of fresh unstained and unfixed virus was recorded photographically under the phase contrast microscope and studied in replicate Giemsa-stained smears.

Phase contrast studies.

Up to 16 hr. after infection no general changes in the host cells could be detected, the nuclei, nucleoli and mitochondria were normal and no virus particles could be recognised with certainty. From 16 hr. onwards it was possible to find within the cytoplasm of some of the mononuclear cells spherical bodies 1·0–2·5 μ diameter; sometimes compact clumps of between 4 and 8 of these bodies were present but more usually they were dispersed in the cytoplasm (Fig. 1).

When infection had continued for 20–24 hr. the virus particles had increased considerably in number and had assumed varying sizes ranging between 0·5 and 1·5 μ in diameter; they were for the most part scattered unevenly throughout the whole cytoplasm of the cell (Fig. 2). In about half of the infected cells, however, the particles were massed closely together to form a colony of the virus which appeared to be surrounded by a well defined envelope. Within the colony, constrictions and indentations in the outlines of the larger particles strongly suggested that active division was proceeding (Fig. 3).

From this time onwards the colonies increased in size and although the particles became smaller it was always possible to distinguish considerable variation in their size. On infrequent occasions two or even three colonies could be seen within the cytoplasm of a single host cell. Between 24 and 36 hr. after infection the virus particles, by this time 0·5 μ or less in diameter, were undergoing rapid Brownian movement within the colony envelope. Sometimes colonies of this type could be seen extracellularly both in fresh preparations under the phase contrast microscope and in stained smears; it was presumed that they had escaped from the host cells as a result of trauma during preparation.

The matrix of colonies 24 hr. old was rather opaque and the virus particles were motionless, while in colonies 36 hr. old and of greater size it became progressively more transparent until the stage of Brownian movement was reached. It thus appeared that concurrently with virus multiplication alterations were occurring in the viscosity and refractive index of the matrix. It was however always possible to find localised collections of virus particles in which no trace of a membrane or matrix could be detected and it was felt that this structure was not a constant feature of the reproductive process. Studies of stained films showed that the matrix does not react with Lugol's iodine and that it takes a faint pink colour with Giemsa.

When infection had continued for 48 hr. or longer no sign remained of any envelope containing the elementary bodies. Brownian movement had ceased. The cytoplasm of the host cells was filled with large numbers of elementary bodies all more or less uniform in size (Fig. 4). Meanwhile the host cells themselves

manifested evidence of damage, the mitochondria were not visible, the nucleus became increasingly transparent, and nucleoli could no longer be distinguished. Vacuolation of the cytoplasm was often prominent. Finally the cell membrane, which was visible throughout, ruptured and the elementary bodies were set free. These findings are in close accord with those of Bland and Canti and also with the observations of Salaman (1953) who described the reproductive cycle of the virus as he saw it in whole mounts of amniotic membranes of infected eggs by means of phase contrast and ultraviolet light microscopy.

Electron microscope studies.

Since yolk-sac cultures alone provided a sufficient yield of the larger virus particles they were used as the source of material despite the disadvantage that the morphological sequence of changes in the growing virus is less clearly demonstrable in the egg than in the tissues of the mouse.

However between 36 and 54 hr. after inoculation some 20 per cent of the yolk-sacs were found to contain considerable numbers of the large form of the virus as judged from impression smears stained by Castaneda's method (Fig. 5). By using sufficiently large batches of eggs it was therefore possible to obtain enough material for purification. Similarly between 54 and 68 hr. after inoculation, virus particles of a range of intermediate sizes were found to predominate especially at a time when the embryo was obviously affected by the virus. When the yolk-sacs of embryos dying some 72 hr. after inoculation were examined the virus was present in the form of elementary bodies of more or less uniform size (Fig. 6).

In every specimen examined varying numbers of elementary bodies were found. These appeared as rounded particles and measurements of 50 show that they vary in diameter between 400 and 500 mμ, the average being 450 mμ. Their appearance is that of spheres which have collapsed as a result of drying, leaving a heaped up central mass of protoplasm and a thin flattened peripheral rim. The whole particle is covered by a rather thick membrane with a wrinkled and folded surface. At the edge of the particle two layers of the membrane are in contact and form the rim (Fig. 7).

Intermediate-sized particles were obtained from 66 hr.-old yolk-sac cultures; their diameters range from 0·50 to 0·80 μ with an average of 0·61 μ. Many of these particles closely resemble the elementary body and less than half of them show any changed structure (Fig. 9). In the larger particles the protoplasm is condensed into ill-defined multiple masses about 0·15 to 0·20 μ in diameter and the limiting membrane is not so clearly defined. Thus a suggestion of division by fission is produced.

In 15 of the large virus particles the diameter ranged between 0·85 and 1·40μ with an average of 1·12 μ. In the largest of these particles, which were obtained from 54 hr. cultures, no internal structure could be defined. They appeared as flattened discs of structureless material whose irregular edges suggest that no rigid covering membrane is present (Fig. 8). Slightly smaller particles with diameters in the region of 1·00 μ were seen to contain small spheres 0·10–0·15 μ in diameter within a very thin covering membrane. In some instances, due no doubt to trauma in preparation, these particles are ruptured. Such a particle is seen in Fig. 10 where the membrane has been broken and a great part of the contents of the particle appears to have escaped; several small rounded particles

however remain within the membrane. Similar small particles separate from recognisable virus particles are seen in Fig. 8 and 9.

Particles of this size (0·1–0·15 μ) are frequently found in normal egg fluids (Ghosh Ray and Swain, 1954) and thus their significance may be questioned. However the disposition of these particles within the membrane of large forms and the fact that they could not be detected in control preparations suggest that they may originate in the virus itself. Moreover their clear-cut edges and general appearances differ appreciably from those of the particles of normal egg fluids.

DISCUSSION.

Studies of the morphological sequence of changes in the microscopical appearances of psittacosis virus during its multiplication described by previous workers and here corroborated by phase contrast photo-micrography indicate that the growth process may be described in four overlapping stages.

In the first stage the scanty numbers of virus particles can scarcely be found microscopically, in the second a few large particles 1–2 μ in diameter are present in the cytoplasm, in the third increasing numbers of intermediate-sized particles are seen sometimes in the form of a virus colony with a matrix and a limiting membrane, and in the fourth stage large numbers of elementary bodies of uniform size are dispersed evenly throughout the host cell.

It would appear that division of the particle takes place at least twice during reproduction since the intermediate-sized particle is approximately one half the size of the large form and the elementary body is nearly half as small again.

In studies of reproduction of bacteria by binary fission the measurement of the size of the multiplying organisms and the study of their changing internal structure has yielded important results. With viruses such information can only be obtained with accuracy by electron microscopical methods. Many technical disadvantages hinder the observation of the consecutive alterations in the contents of infected cells in the electron microscope. The observations of Kurotchkin, Libby, Gagnon and Cox (1947) and of McFarlane (1949) lack a correlation of time with the sequence of the morphological changes of the virus, and the electron micrographs of Heinmets and Golub (1948) show particles which may have been distorted by the ultra-sonic vibration used in the preparatory work.

In the present electron microscopical studies three forms of the virus have been distinguished during the active phase of its multiplication. The earliest is the large form whose average diameter is 1·20 μ and which has a very thin and poorly developed covering membrane. Next follows an intermediate-sized particle with an average diameter of 0·62 μ in which multiple discrete masses of protoplasm have been formed. The final stage is characterised by mature elementary bodies of uniform size varying in diameter by only 0·05 μ from a mean of 0·45 μ; they have a thicker and stronger covering membrane and show no sign of further division by segmentation of the protoplasm.

The progressive diminution of the size of the virus particles and the alteration in their structure during rapid multiplication are analagous to the morphological changes which occur in the bacterial cell during the late lag phase and the logarithmic phase of rapid growth. The affinity of the bacterial cell for basic dyes alters during active division and so also do the staining properties of the psittacosis virus particle during the early stages of its reproduction (Bedson and Bland, 1932).

Another member of the psittacosis group, meningopneumonitis virus, has been studied by Gaylord (1954) in thin sections of the chorio-allantoic membrane at intervals after infection. This author has recognised a large initial body of the virus similarly related in time sequence to intermediate-sized particles and to elementary bodies. He has also described a single membrane covering the large particles, two or three membranes enclosing the intermediate-sized particles, and very dense central granules within the elementary bodies.

Although Gaylord has agreed that reproduction by binary fission does occur, he has introduced the concept that reproduction by endosporulation also operates and that elementary bodies represent a spore-like stage. To support this contention he has drawn attention to yeast-like buds which are to be seen in one of his electron-micrographs and to multiple masses within the large forms. The diameter of the " buds " and the intermediate-sized virus particles is of the same order. However, it is not possible to be certain whether these " buds " are truly attached to the initial bodies or whether the appearance has been produced by compression of the microtome knife or is an artefact due to the angle of cutting. " Buds " of this type were never encountered in the present work nor were they observed by earlier workers on psittacosis virus.

It may be seriously doubted whether it is biologically sound to postulate two entirely distinct means of reproduction for the same organism, especially for one so little differentiated as a virus. The step-like diminution of particle size of both the psittacosis and meningopneumonitis viruses is linked in time sequence with active multiplication. The process is most satisfactorily explained as one of simple binary fission. The small bodies within the large forms of psittacosis virus

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EXPLANATION OF PLATES.

FIGURES 1–4 are phase contrast photomicrographs of wet films of fresh unfixed cells from the spleens of mice at intervals after infection. All × 1080.

FIG. 1.—Sixteen hr. after infection. Shows eight large virus forms dispersed in the cytoplasm of the host cell.

FIG. 2.—Twenty hr. after infection. Medium-sized particles with considerable differences in diameter.

FIG. 3.—Twenty-four hr. after infection. A colony of virus particles of varying sizes embedded in a matrix. One large particle appears to have divided in four and another in two.

FIG. 4.—Forty-eight hr. after infection. The virus is entirely in the form of elementary bodies dispersed in the cytoplasm.

FIGURES 5–6.—Impression smears of yolk-sac cultures. Castaneda's stain. × 1150.

FIG. 5.—Forty-eight hr. after infection. Large forms predominate.

FIG. 6.—Seventy-two hr. after infection. A cluster of elementary bodies.

FIGURES 7–8.—Electron micrographs of purified virus suspensions from yolk-sac cultures. × 20,000.

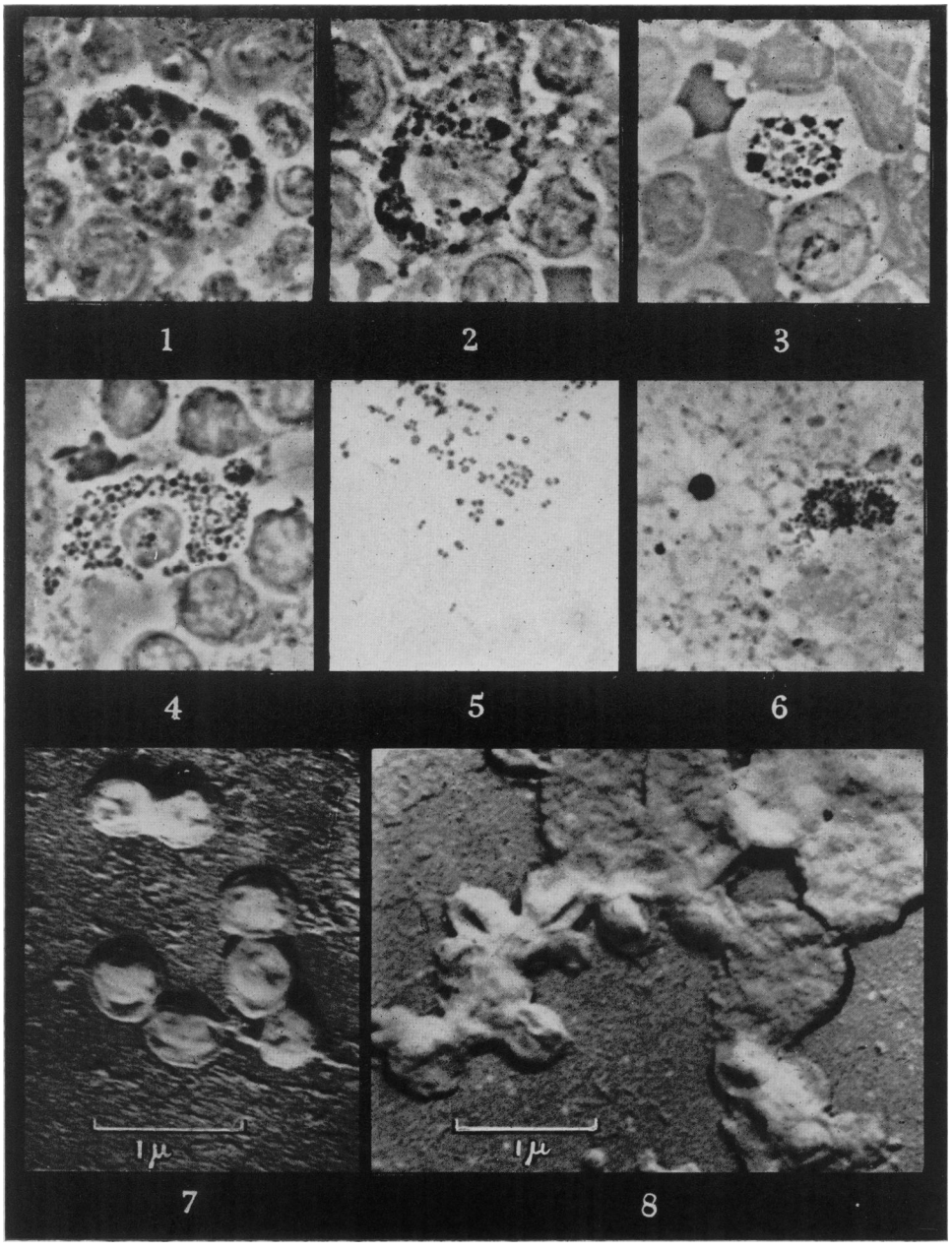
FIG. 7.—Seventy-two hr. after infection. Elementary bodies.

FIG. 8.—Fifty-four hr. after infection. Large forms together with elementary bodies. A small particle 0·10 μ in diameter is seen at the lower margin.

FIGURES 8–9.—Electron micrographs of virus suspensions purified from yolk-sac cultures. Both × 20,000.

FIG. 9.—Sixty-six hr. after infection. Elementary bodies and intermediate-sized particles, the larger of which contain several masses of protoplasm.

FIG. 10.—Fifty-four hr. after infection. Large virus particles with smaller forms. One large particle shows a broken covering membrane and appears to have been emptied of a part of its contents. Several discrete masses are seen within the membrane.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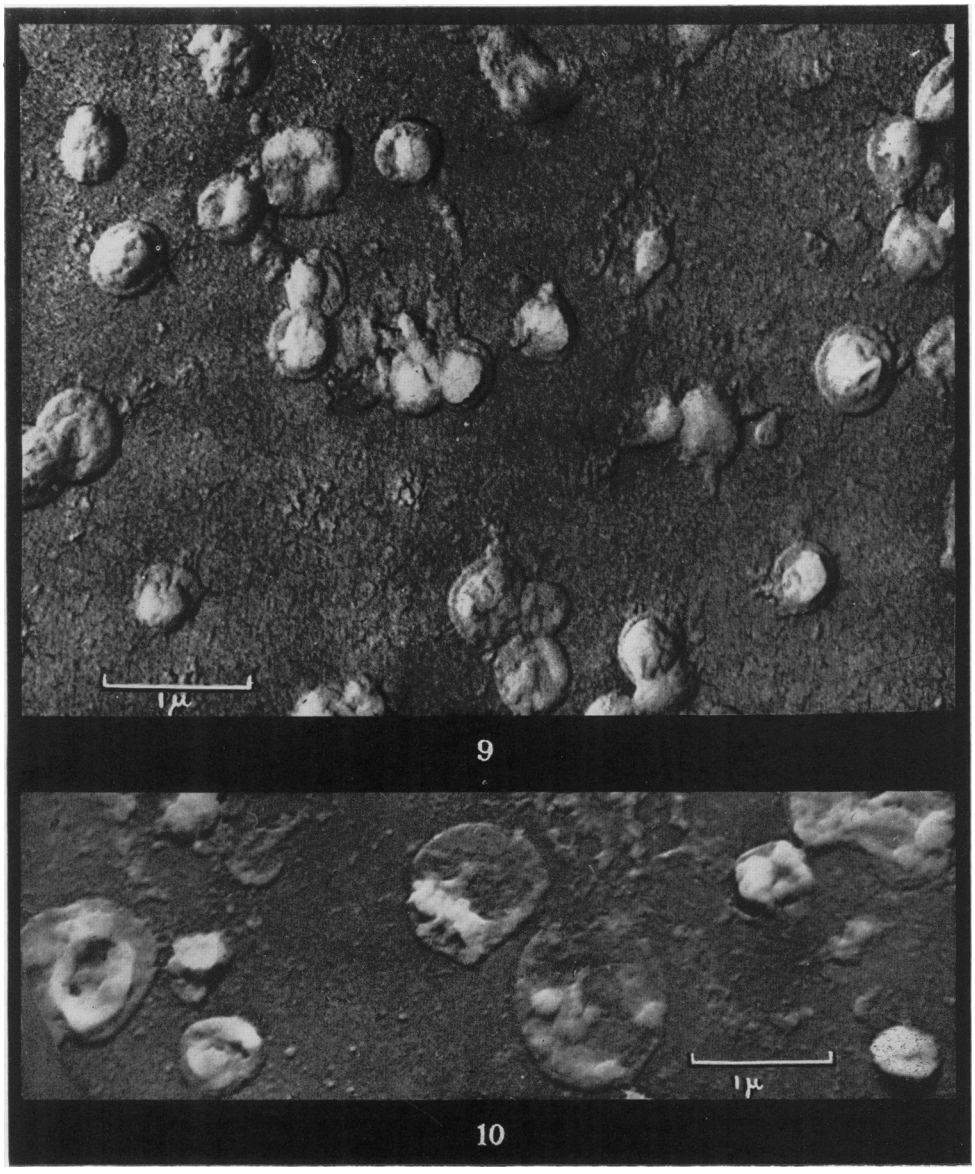
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seen in Fig. 9 and noted in the meningopneumonitis virus by Gaylord may be no more than nuclear and cytoplasmic condensations preceding division. Such masses occur in bacteria during active increase by binary fission and the observation hardly provides sufficient evidence on which to base a hypothesis of reproduction by multiple endosporulation.

If an organised colony with a well-defined architecture were an invariable and essential feature of a life cycle of psittacosis virus, there would be some difficulty in accepting the hypothesis that its reproduction is by a process of binary fission. Since, however, it is often possible to find clumps of virus particles of all sizes, without any suggestion of a matrix or covering membrane, it would seem likely that organised colonies may only occur under special circumstances. The matrix may be derived from the cytoplasm of the host cell as a reaction to the presence of the growing virus and the membrane may consist of compressed protoplasm.

An alternative explanation of the nature of the supporting structure of the colony is that it is formed by a secretory product of the virus itself. This may be true of molluscum contagiosum where van Rooyen (1939) has shown that the inclusion is covered by a membrane which, on account of its staining properties with iodine, may be composed of a carbohydrate substance. Such however is not the case with the colony of psittacosis virus of which the staining of the matrix differs only very slightly from that of the host cell.

It is of interest to note the close morphological resemblances of the elementary bodies seen in these electron micrographs to those of feline pneumonitis virus reproduced by Hamre, Rake and Rake (1947) and by Moulder (1954) where similar size variations are apparent.

SUMMARY.

The results of a phase contrast photomicrographic study of the reproduction of psittacosis virus in the spleens of mice inoculated intraperitoneally have been compared with electron micrographs of the virus obtained at timed intervals during its multiplication in the yolk-sac of the chick embryo.

Measurements of the sizes of the various forms of the virus show a decrease in the particle size with the lapse of time. Concurrently a limiting membrane is developed and the protoplasm of the virus particle is condensed into discrete masses.

It is concluded that the evidence presented supports the view that, during the active phase of its reproduction, psittacosis virus divides by binary fission.

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