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THE INFECTIVITY OF ISOLATED INCLUSION BODIES OF FOWL-POX *

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The group of so-called filterable virus diseases is coming to occupy an increasingly important place in medical and biological literature. Included in this group is fowl-pox or epithelioma contagiosum of birds.[1] In common with many of the other virus diseases fowl-pox is characterized by the presence of inclusion bodies in the affected epithelial cells. Ever since their discovery[2] the question has been raised with regard to these bodies as to whether they represent a mere degeneration product of the cell,[3,4] or whether, in some fashion, they actually carry the infectious agent of the disease.[5,6]

In recent publications from this laboratory[7,8] morphological evidence has been presented which seems to justify the opinion that the inclusion bodies represent colonies of a minute microörganism (bodies of Borrel[9]) enveloped in a membrane of lipoproteid composition which is semipermeable. The inclusions, when placed in distilled water, swell, with the formation of vacuoles in which numbers of rounded uniform bodies measuring about 0.25 micron in diameter can be seen in rapid Brownian motion. The addition of sodium chloride causes the bodies to shrink and to assume again their initial hyaline, homogeneous appearance. A crushed inclu-

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sion, suitably stained, discloses the minute bodies enclosed in a faintly staining homogeneous material which is regarded as a sort of brood capsular material for a living agent represented by the minute granules. It was in the attempt to shed some added light on the question of infectivity of the inclusion bodies that the following series of experiments was undertaken.

The characteristic lesion of spontaneous fowl-pox is a local epithelial hyperplasia. The virus proliferates locally in these eruptive cutaneous lesions and material from them is highly infectious. So far as determined an increase in virus is limited to these areas of epithelial hyperplasia. Only squamous epithelium of the skin and mucous membranes seems to be susceptible to the virus. Experimental infection is readily induced by applying active virus to any superficially injured squamous epithelial surface. If a feather be plucked from the breast of a hen and the follicle inoculated with material from an eruptive lesion a slight swelling of the follicle may be noted after three days. After seven to ten days the follicle has increased to about ten times its original size. This is shown in Fig. 1 in which the follicle "*a*" had been inoculated ten days previously. The other follicles remain normal. By carefully dissecting away the skin the swollen epithelial core of the follicle may be shelled out as shown in Fig. 2. This particular core is about 1½ mm. in diameter and about 3 mm. long. If a section be made of it the core is found to consist of concentric layers of swollen epithelial cells in each one of which may be seen one or more of the inclusion bodies (Fig. 3). The swelling of the follicle is due both to an hyperplasia of the epithelium and to a swelling of the individual cells in the stratum mucosum. The cells in this region are enlarged to several times the size of those in the stratum germinativum. The eosin-staining inclusion body is usually in the center of the cell, with a broad, non-staining zone between the inclusion and the cell membrane. The bodies may also be distinctly seen within the epithelial cells in a fresh preparation of the diseased tissue teased out in saline.

The question regarding the nature of the inclusion bodies in fowl-pox has been approached from a new angle through the discovery that, using a 1 per cent solution of trypsin in 0.2 per cent sodium bicarbonate, the cellular material of a fowl-pox lesion may be digested away completely leaving the inclusion bodies free. This process of freeing the inclusion bodies by digestion is a striking

thing to watch. If the core of epithelium just illustrated be cut into small pieces and placed on a glass slide along with a few drops of trypsin the inclusion bodies begin to show more distinctly almost immediately and, after five minutes in the solution, many of the bodies drop free from the tissue to lie like minute pearls on the surface of the slide (Fig. 4). After thirty minutes almost all of the inclusion bodies are free as shown in Fig. 5, and the amorphous mass of digested epithelium and connective tissue may be shaken about with a needle and separated completely from any remaining inclusion bodies.

The way in which the epithelial cells of a fowl-pox lesion are digested away completely, leaving the inclusion bodies unchanged, in itself suggests that these bodies represent something more than a degeneration product of the cell. As shown in Fig. 6, taken forty-five minutes after the beginning of digestion, the inclusion bodies remain intact while all that remains of the epithelium is the amorphous material seen as the groundwork of the picture.

The material which may protect the inclusion bodies from tryptic digestion is a fatty element readily demonstrable by means of special fat stains. In addition to the fat there is an albuminous component of the body.[10] The structures are elastic and may be indented with a needle only to regain their shape as soon as the needle is removed. In general the bodies are rounded or oval as shown in Fig. 7. Occasionally one may be bean-shaped or otherwise irregular. The irregularity in shape occurs usually in the larger bodies, the smaller ones being almost always rounded. In size the bodies vary from 2 or 3 microns to perhaps 50 microns in diameter. They are of relatively high density and quickly gravitate to the bottom if suspended in a physiological saline solution. They may be readily rolled about in saline on a glass slide though an occasional body may adhere to the slide and leave a small portion of its substance if broken away. The bodies are highly refractive. As shown in Fig. 7 they look, under the higher magnification of the microscope, like miniature white potatoes.

The compact nature of the inclusion bodies when freed by digestion makes them very easily manipulable for the purpose of inoculation. The trypsin may be pipetted from the surface of the bodies and be replaced with saline. This in turn may be pipetted off and the process repeated until the bodies appear to be free from débris.

After each addition of saline the bodies may be agitated in the solution since they rapidly settle to the bottom again. Those bodies which happen to strike the surface of the solution form an exception to the rule for, once on the surface, they are closely held there by surface tension. After some experimenting it was found that a 2 per cent salt solution seemed to keep the bodies slightly more firm and dense than physiological saline, so the 2 per cent solution was used exclusively for the fluid medium in our experiments.

After freeing the inclusion bodies from all cellular material an attempt was made to test their infectivity. Former attempts in this direction have been hampered by the failure to get the bodies cleaned sufficiently from their cellular surroundings. The process of digestion encouraged us in the belief that the inclusion bodies could be freed and washed clean enough to make their inoculation of value in determining whether or not the inclusion carries the infective agent of the disease. Among all of the inclusion body diseases, fowl-pox lends itself with particular readiness to this experiment because of the large size and the compactness of the inclusions. Furthermore a bird is inoculated with extreme ease since the simple operation of plucking a feather leaves an epithelium-lined follicle which is an ideal nidus for the inoculation of minute amounts of fluid.

For picking up the individual inclusion bodies a Chambers microdissection apparatus was found necessary. Two variations from the usual Chambers technic for isolating single bacteria by the pipette method were employed. Instead of using a hanging drop it was necessary to work with a small pool of material on the upper surface of the slide. This was because the inclusion bodies in the hanging drop fell to the dependent surface of the fluid, and once they had come under the influence of surface tension it was found impossible to remove them from the fluid. The second change found necessary was the introduction of a micro-adjustment for the plunger of the injection syringe. This syringe, when worked with the unaided hand, forced a veritable torrent of fluid through the opening of the fine capillary pipette to which it was attached. With a simple screw attachment for the plunger it was found possible to regulate perfectly the flow of fluid through the capillary opening.

The apparatus is used under the low power of the microscope with the light cut down so that the inclusion bodies show up promin-

ently on a dim field. The digesting and washing are done with the help of a dissecting microscope.

In the first inoculation experiment a small piece of infected epithelium was digested in trypsin for one and a half hours. The inclusion bodies were then washed with saline. About a hundred, as seen under the dissecting microscope, were sucked into a large pipette, transferred to another slide, and this slide was introduced under the microscope with the Chambers attachment. From five to ten inclusion bodies were sucked into a fine capillary pipette and inoculated into a breast follicle from which the feather had been plucked. Since the object of our experiment was to find whether the virus is concentrated in the inclusion or in the cellular material about it, the inoculation of inclusion bodies was controlled by the inoculation of the fluid overlying them into a follicle on the opposite side of the chicken. This fluid was by no means clear but contained numerous small particles floating in it. Consequently, when after seven days a bilateral "take" occurred on the chicken, it was thought that possibly a small inclusion body or portion of inclusion had been contained in the supernatant fluid of the control inoculation.

A refinement of technic was next attempted by picking up five or ten inclusion bodies in the capillary pipette with a minimum of fluid, transferring these to an entirely new pool of saline and from this pool picking up the inclusion bodies with a fresh pipette for inoculation into the fowl. However, this experiment too showed a "take" for both the inclusion body inoculation and for the supernatant fluid.

In the hope that a further washing of the inclusion bodies might give a supernatant fluid free from virus the bodies were next passed through two distinct pools of saline. A sample protocol of the digesting and washing process employed may be roughly diagrammed as follows, using Hen P as an illustration.

1 {Digesting pool under dissecting scope} Several hundred inclusion bodies transferred to

2 {Sorting pool under Chambers scope} 21 inclusion bodies picked up 14 delivered to

3 {First wash pool} 7 inclusion bodies picked up 4 delivered to

4 Second wash pool — 4 inclusion bodies inoculated into anterior breast follicle, right side of Hen P.
Supernatant fluid inoculated into anterior breast follicle, left side of Hen P.

The amount of fluid sucked into the capillary pipette in the transfer from Pool 2 to Pool 3 and again from Pool 3 to Pool 4 was in each instance not over .01 cc. Pools 3 and 4 each consisted of at least 1 cc. of saline, so that the fluid carried over from Pool 2 was diluted 1 to 100 in the first transfer and again 1 to 100 in the transfer from Pool 3 to Pool 4, making a final dilution of at least 1 to 10,000 of any fluid carried over from Pool 2.

In three different experiments chickens were inoculated on the right breast with inclusion bodies washed in this fashion and on the left breast with supernatant fluid equal in amount to the fluid inoculated with the bodies. The results of these experiments were uniformly positive on the side inoculated with inclusion bodies and negative on the side inoculated with their supernatant fluid. The diagnosis of a positive result was based on a demonstration of inclusion bodies in the lesion and on its content of active virus as determined by inoculation.

Chart Showing Results of Inoculations with Washed Inclusion Bodies

Follicle Number	Number of Bodies Inoculated	Result	Control
N1	6	+	−
O1	2	+	−
P1	4	+	−
K1	1	−	−
M1	1	+	*
T1	1	−	−
T2	1	+	−
U1	1	−	−
U2	1	−	−
U3	1	−	−
V1	1	−	−
V2	1	−	−
V3	1	−	−
W1	1	+	−
W2	1	+	−
W3	1	−	−
WH1	1	+	−
WH2	1	+	−
WH3	1	+	−

* No control

The question next arose as to how many inclusion bodies must be inoculated in order to secure a "take." In the preceding experi-

ments the average number of inclusion bodies inoculated into each follicle had been four. A series of sixteen follicles was next inoculated with one inclusion body in each follicle. As shown in the chart seven of these inoculations showed a "take" while nine were negative. In no instance, however, was a single control inoculation positive.

A probable explanation of the failure to secure positive results in a larger percentage of the single inclusion body inoculations seemed to lie in the difficulty experienced in delivering a single body from the pipette into the follicle. As graphically illustrated for Hen P only fourteen of the twenty-one inclusion bodies picked up could be delivered into the first wash pool and only four out of seven could be delivered into the second wash pool. This example is characteristic of our experience in all of the attempts to transfer the inclusion bodies from one pool to another and indicates a propensity of the bodies for sticking to the wall of the pipette. Considering this difficulty the low percentage of "takes" is not remarkable. There was no gradation in the size or in the time of appearance of the "takes," those which appeared at all coming on just as quickly as those which had been inoculated with four or five inclusion bodies.

The lesion caused by the inoculation of one inclusion body is shown in Fig. 8. Each of the two swollen follicles was inoculated seven days previously with a single inclusion body. The swollen follicles are 2 to 3 mm. in length and contain several hundred thousand inclusion bodies similar to the single inclusion body with which each was originally inoculated. The anterior one of these two follicles was removed and digested so as to free the bodies. The digestion mixture was then smeared on the plucked and scarified breast of a chicken. The lesion resulting after seven days is shown in Fig. 9. The massive "take" in the plucked follicles and along the lines of scarification is, then, the result in two weeks time of the inoculation of a single inclusion body.

Discussion

The rapid development of a lesion following inoculation with a single inclusion body we have interpreted as being due to the inoculation not of a single microörganism but of a large number enclosed within the inclusion. Special stains of crushed inclusions show by

count hundreds of definite, rounded objects about 0.25 micron in diameter within each body.[7] These minute objects are believed to represent the actual virus of fowl-pox. Consequently, in inoculating a single inclusion body, one is probably inoculating an entire colony of virus bound up in its fatty capsule.

Working with the digested material from a fowl-pox lesion gives one a very definite impression as to the character of the inclusion bodies. The way in which the bodies resist tryptic digestion marks them as something more substantial than the cell which contains them. The way in which the bodies can be washed clean and inoculated to give a "take," while their supernatant fluid remains sterile, indicates that the inclusion bodies actually carry the infectious agent of the disease. The way in which a single inclusion body, when inoculated, produces in two weeks time a lesion containing millions of similar bodies indicates that the inclusion bodies of fowl-pox contain and are caused by a living, growing virus. So certain have we become that the inclusions carry a virus that the bodies are now spoken of with confidence as *virus* bodies rather than inclusion bodies.

The results of these experiments afford additional evidence that the typical lesion of fowl-pox is induced through an invasion of epithelial cells by the virus and its intracellular proliferation locally within the lesion.

Summary

1. The inclusion bodies of fowl-pox are composed of hundreds of minute bodies enclosed in a fatty capsule.

2. The hyperplastic epithelium of the lesion of fowl-pox, when subjected to tryptic digestion, liberates the intact inclusion bodies while the epithelial cells undergo complete digestion.

3. A single inclusion body, when washed with saline and inoculated into the skin of the hen, has produced a typical fowl-pox lesion containing the characteristic inclusions. The fluid in which the inclusion body is finally suspended is innocuous.

4. The inclusion bodies of fowl-pox are interpreted as being true *virus* bodies, *i.e.*, minute colonies of the etiological agent of the disease.

5. The proliferation of the virus is accordingly largely, if not entirely, intracellular.

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DESCRIPTION OF PLATES

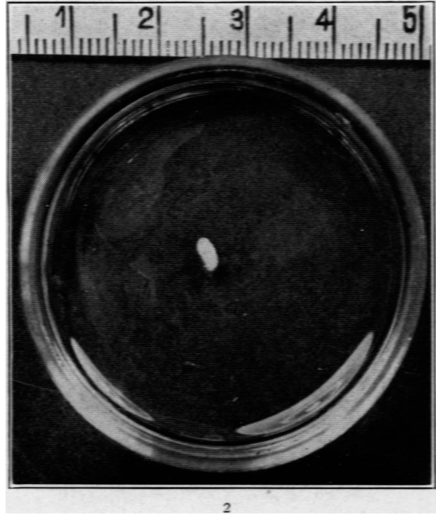
PLATE 1

FIG. 1. Swollen feather follicle at "*a*," ten days after inoculation with a single inclusion body of fowl-pox.

FIG. 2. Epithelial core of inoculated feather follicle. This particular core was removed from the anterior follicle illustrated in Fig. 8.

FIG. 3. Section through feather follicle removed ten days after inoculation with fowl-pox. The dark bodies within the swollen epithelial cells are the inclusion bodies. Hematoxylin and eosin. × 80.

1



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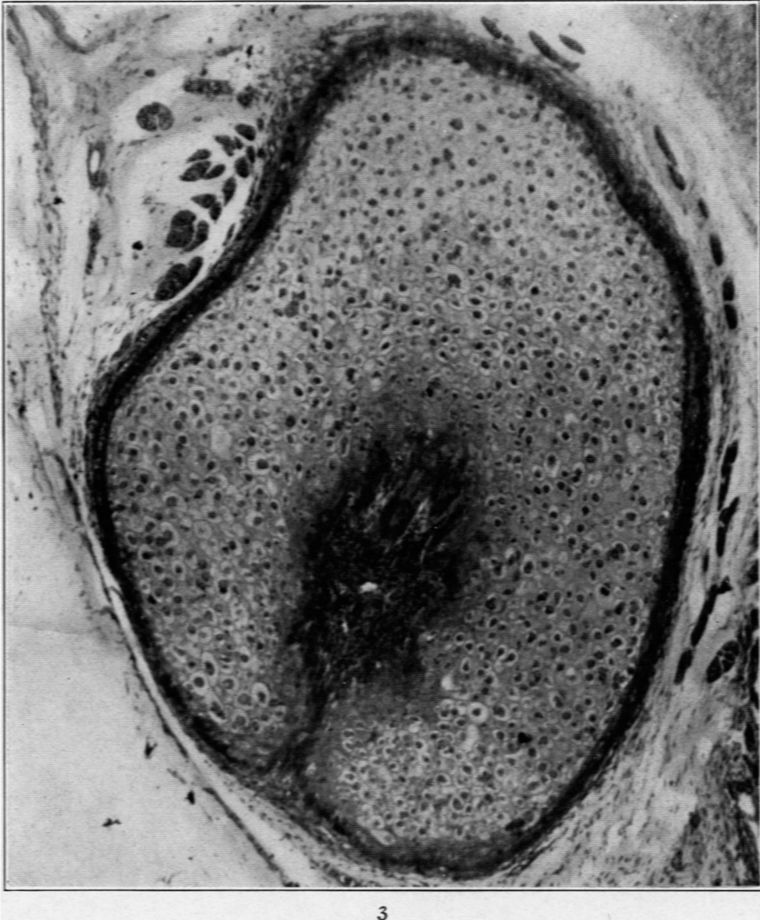

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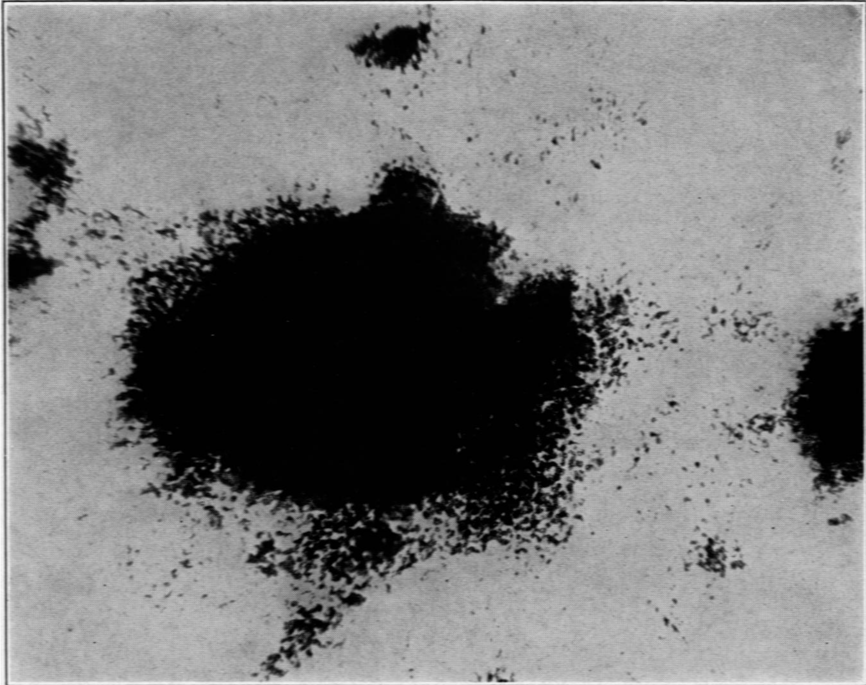
PLATE 2

FIG. 4. Mass of infected epithelial cells in the process of digestion. Some of the freed inclusion bodies may be seen on the right-hand edge of the epithelial mass. $\times$ 47.

FIG. 5. Inclusion bodies thirty minutes after the beginning of digestion. $\times$ 160.



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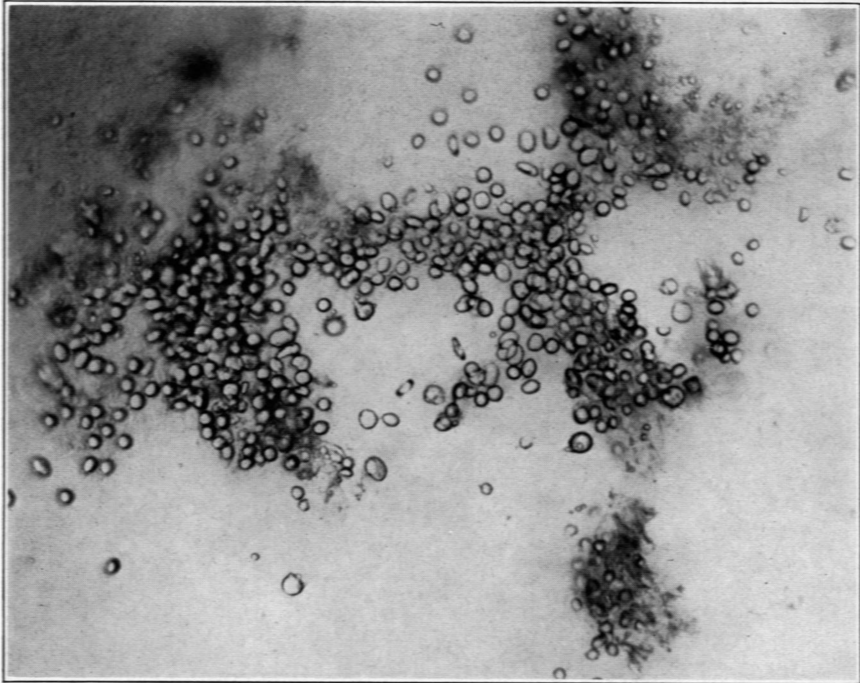

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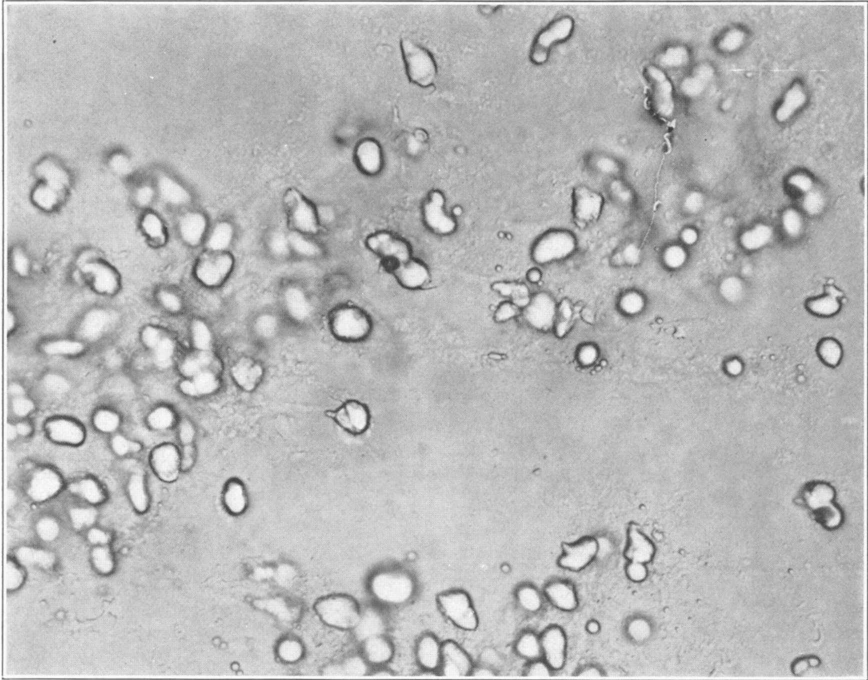
Plate 3

Fig. 6. Inclusion bodies forty-five minutes after the beginning of digestion. Amorphous material forming the groundwork of the picture represents the digested epithelium. × 330.

Fig. 7. High magnification of inclusion bodies completely freed by digestion. × 1000.



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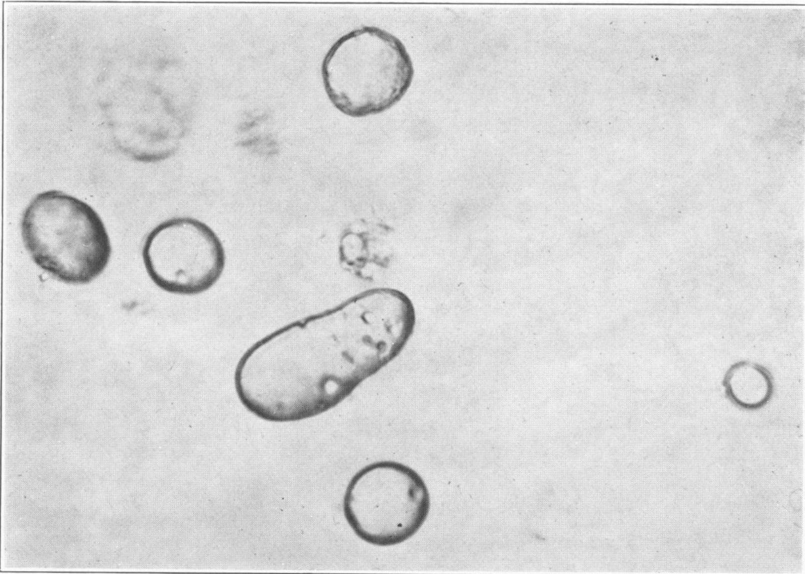

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Plate 4

Fig. 8. Two feather follicles each of which had been inoculated seven days previously with a single inclusion body.

Fig. 9. Plucked and scarified breast of hen seven days after inoculation with inclusion bodies from the anterior follicle illustrated in Fig. 8.

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