STUDIES ON LYMPHOGRANULOMA VENEREUM

I. Development of the Agent in the Yolk Sac of the Chicken Embryo

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Plates 9 to 11

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The demonstration that the method of yolk sac inoculation, first used so successfully by Cox (1) in his investigations on *Rickettsiae*, could be applied with success to the growth of the agent of lymphogranuloma venereum (2) has stimulated more extensive investigations of this disease and its causative agent (3–12). Amongst other studies which have been made is one of the nature of the development of the agent in the cells of the yolk sac. This has been the subject of a preliminary communication elsewhere (13). The purpose of the present paper is to describe this development in detail with regard both to morphological changes and to concomitant changes in the infective titre of the yolk sac.

The first description of apparently specific inclusions in connection with lymphogranuloma venereum was given by Gamna in 1923 (14) and again in 1924 (15) when the observations were confirmed by Favre (16). Both these observers noted in mononuclear cells from infected lymph nodes, whether in the natural human disease or in experimental disease in guinea pigs, cytoplasmic inclusions varying in size from those which were barely visible to others 4.5μ in diameter. These might be multiple in one cell, varied in size, and were often either vacuolated or contained an acidophilic center and basophilic periphery. They were not found in other conditions or in normal lymph nodes. Similar bodies were seen in lymphogranuloma venereum by Todd (17). In publications in 1933 (18) and 1938 (19) Findlay cast doubt on the virus nature of these bodies because many of them give a positive reaction for thymonucleic acid, but in retrospect it seems probable that they do represent different stages in the development of the agent.

In 1927 Gay-Prieto described small bodies which seemed to be specific (20). These appeared both within and between the monocytes surrounding the abscesses; they were of 1μ or less in size; were mostly round, oval, or semilunar; and were metachromatic. Twelve or more might appear in the cytoplasm of a single cell. In 1933 Findlay (18) also described bodies similar to those of Gay-Prieto. These were found in human material and also in the experimental disease in monkeys, guinea pigs, and mice. They were from 1 to 4μ in diameter and were found in lymphocytes, in plasma

cells, or lying free. They were basophilic and the smaller ones sometimes occurred in large masses.

In 1935 Miyagawa and his associates described for the first time "granulo-corpuscles" about 0.3μ in diameter which resembled closely the elementary bodies of other viruses (21). These bodies were found in monocytes, leucocytes, and glial cells in the brains of intracerebrally infected monkeys. They were solitary, in pairs, or in groups; did not give a Feulgen reaction; and were negative with Gram stain. Similar bodies were found in all human material from cases of lymphogranuloma venereum. In a subsequent paper (22) similar bodies were described in monocytes, glial cells, and neurones of intracerebrally infected mice. In advanced lesions they increased markedly in number so that they came finally to fill the infected cell. Although not mentioned in the text, the illustrations for both these papers suggest that the specific bodies may vary in size. The presence of these bodies was soon confirmed by other investigators, for example Nauck and Malamos (23), who noted that they resembled closely the elementary bodies of psittacosis in their staining reactions with Giemsa stain. In the preparations of these authors paired forms were common. The elementary bodies often occupied vacuoles in the cell cytoplasm or were embedded in a (with Giemsa) reddish violet matrix.

Several filtration studies with Elford gradocol membranes were carried out by various investigators and gave results in keeping with the microscopic findings of Miyagawa and his associates (21). Thus by such filtration Broom and Findlay arrived at a size of from 125 to 175mμ for the elementary bodies (24).

Differences in size of the virus bodies and in their staining reactions soon were noted (25, 26), and in 1938 Findlay and his colleagues suggested that the virus might undergo a developmental cycle (27). In the early lesions in intracerebrally infected mice large bodies were seen 0.4 to 0.7μ in diameter. These stained bluish violet with Giemsa and were often irregular in shape, suggesting fission. Intermediate forms between these and the reddish violet elementary bodies of 0.1 to 0.175μ were seen. The large forms occasionally occurred massed together into plaques. Malamos (28) also noted larger blue-staining bodies in the early stages of the infection of tissue cultures of rabbit corneal epithelium. These larger bodies increased in size and, while homogeneous at first, were later seen to contain elementary bodies. These larger plaques became vacuolated and might break up into shreds and uneven fragments. In a later study Findlay and his colleagues gave a more detailed description of the suggested cycle of development of the agent in the brains of infected mice (19). Following intracerebral inoculation no virus could be seen in smears for some hours. The initial bodies which were seen were larger than elementary bodies. Since dumbbell-like division forms were noted it appeared that these large bodies divided. Compact masses of large bodies might result from which individual bodies might break off, enter other cells, and give rise to elementary bodies but no decision was reached as to whether the elementary bodies themselves divided, although pairs and short chains were noted. When sufficiently numerous in the cytoplasm, the elementary bodies formed collections within vacuoles with definite limiting membranes; the membranes might eventually rupture and liberate the elementary bodies which could then infect new cells.

Amongst others who have noted more than one form of the virus bodies are Schoen

(29), and Gey and Bang (30). Schoen (29) noted that the first bodies to be seen inside vacuoles in the ependymal cells of infected mouse brains at 48 hours were 2 to 3μ. By 4 days the bodies had become smaller and might be bacilliform. Gey and Bang (30) used a tissue culture of fibroblasts from human thyroid and grew the virus for as long as 7 months. After 7 days small vesicles were seen in the fibroblasts and these later increased in size. The small vesicles contained bodies about 1μ in diameter but the larger vesicles, which occupied the whole cell, were filled with bodies 0.2μ in diameter which showed brownian movement. No large inclusions other than the vesicles were seen. The latter were thought to represent response of the cells to the presence of the virus.

Technique

All the studies of development described below have been carried out with one strain of lymphogranuloma venereum, namely that obtained originally through the courtesy of Dr. W. L. Fleming and termed by us J. H. However, similar morphological elements have been seen in the other three strains with which we have worked, one of which was isolated by inoculation of human pus into the yolk sac.

All inoculations have been made into the yolk sac of 6 day old chicken embryos (1) and have been of 1 ml. volume. Suspensions have been made by shaking infected yolk sacs for 30 minutes on a machine with sufficient beef heart infusion broth (pH 7.6) to produce a 10 per cent dilution. These yolk sacs had been freed of most of their yolk by careful draining but had not been washed. Throughout the investigation the titre of the virus has remained almost unchanged both for embryos by the yolk sac route with an L_D 50 of 10^{-9}, and for mice by the intracerebral route with an L_D 50 of 1 to 416. In studying the development of the agent in the yolk cells, different dilutions of infected yolk sac have been used as inoculum, as will appear below, but a dilution of 10^{-1} or 5×10^8 infective doses has given us the most significant results. In titrating the amounts of virus present in the yolk cells at different stages of development, the yolk sac method of inoculation has been used and eggs surviving for 15 days after inoculation (*i.e.* to the point of hatching) have been opened and examined for the presence of virus both by smear and, in many cases, by section. The titres given are therefore those of persistent infection rather than mortality, although in most cases these have corresponded. Of 368 eggs which were opened on the 15th day after infection and found to be alive, 16 or 4.4 per cent have been found by smear or section to be infected. In 567 eggs, whether dead or alive, found negative on smear 42 or 7.4 per cent have been found positive on section. The yolk sac method has also been used in estimating the amount of virus present in embryonic tissues other than the yolk sac.

In the morphological studies use has been made both of smears or impressions and of sections. Smears and impressions are made by smearing or gently impressing on a clean glass slide a small fragment of yolk sac from which excess free yolk has been removed on a gauze sponge. For Gram or Macchiavello stain these smears or impressions have been heat-fixed. For Giemsa stain, whether the slow or the rapid method be used, they have been fixed in 95 per cent methyl alcohol. Material for

paraffin section has been fixed in Zenker's or Helly's fluid. After the former fixation staining has been carried out with eosin and methylene blue or with Giemsa. After fixation with Helly's fluid Noble's stain, as described by Yanamura and Meyer (31), has been employed.

RESULTS

As has been indicated elsewhere (2), during the earlier stages of infection after yolk sac inoculation, virus could be demonstrated, by intracerebral inoculation of mice, only in the yolk sac and to a lesser degree in the yolk. The brain, viscera, and chorio-allantois contained no virus demonstrable by this method. If the infection were permitted to progress until the embryo was moribund or dead and titration were made in the yolk sac results were obtained as shown in Table I. The titre of the original inoculum and the consequent period of survival of the embryos did not appear to be of any great importance as far as the distribution and titre of virus in the moribund or dead embryo were concerned. It will be noted that the yolk contained considerable amounts of virus but always less than that found in the yolk sac, while the embryo itself or its component tissues contained much less. It seemed clear from these results that the multiplication of the virus was occurring principally in the yolk sac and that the virus found in the other tissues of the embryo was due in all probability to invasion of the vessels in the highly vascularized yolk sac tissue with perhaps slight multiplication in favorable sites. The presence of the virus in the yolk was due to the rupture of the infected yolk cells which, as will appear below, were often completely filled with virus bodies. At no time has there been any evidence of multiplication in the yolk itself, and studies have shown that, far from multiplying in yolk removed from the egg, viable virus rapidly disappears and in most cases a starting titre of 10^6 to 10^8 infective units per ml. has fallen to zero in 48 hours.

TABLE I

	Average 50 per cent endpoint of infectivity	Outside limits of titre in different experiments
Yolk sac	$10^{-8.42}$	10^{-7} to 10^{-9}
Yolk	$10^{-7.0}$	10^{-6} to 10^{-8}
Chorio-allantois and amnion	$10^{-1.95}$	10^{-1} to 10^{-4}
Whole embryo less membranes	$10^{-2.77}$	10^{-1} to 10^{-4}
Brain	$10^{-1.24}$	10^{-1} to 10^{-3}
Skin and muscle	$10^{-2.59}$	$<10^{-2}$ to 10^{-4}
Lungs	$10^{-2.25}$	10^{-1} to 10^{-3}
Liver, kidney, and spleen	$10^{-3.75}$	$<10^{-2}$ to 10^{-5}

For the above reasons it seemed logical to confine our investigations of the development of the agent of lymphogranuloma venereum to the yolk sac of

the embryo. The most informative results have been obtained with an inoculum of 5×10^8 infective units, or 1 ml. of a 1 in 10 dilution of a heavily infected yolk sac, and frequent killing of embryos thereafter for both titration and morphological studies. Representative titration curves are shown in Chart 1 which also includes certain important data from the morphological studies. Table II contains the pertinent data.

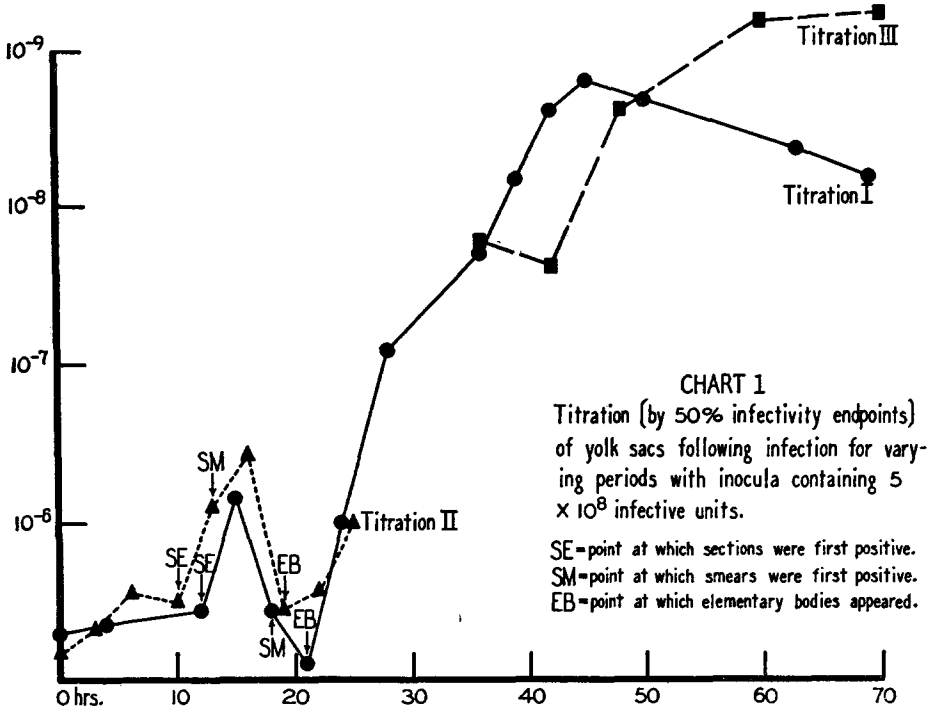

CHART 1

Titration (by 50% infectivity endpoints) of yolk sacs following infection for varying periods with inocula containing 5 × 10^8 infective units.

SE = point at which sections were first positive.
SM = point at which smears were first positive.
EB = point at which elementary bodies appeared.

In both of these studies large numbers of eggs were inoculated by the yolk sac method with 1 ml. of a 10^{-1} dilution of a heavily infected yolk sac. At successive short time intervals thereafter 3 eggs were opened and the yolk sacs removed. Small fragments of each yolk sac were used to prepare smears and impressions; larger fragments were fixed in both Zenker's and Helly's fluids, and the remainders of the yolk sacs (approximately an equal amount from each of the 3 eggs) were pooled and shaken with sufficient beef heart infusion broth to give a 1 in 10 original dilution. From this dilution appropriate other dilutions were prepared and 10 eggs inoculated with each appropriate dilution by the yolk sac technique. On death of any of these test embryos, or after 15 days incubation in the case of those surviving (*i.e.* at hatching time) smears were made from the yolk sacs and these were stained with Giemsa and Macchiavello stains and examined for virus. In those cases in which there remained any doubt after examination of the smears, sections were prepared and examined.

As will be seen from the titrations there was but slight increase in the titre of virus up to 12 hours; during the next 4 hours however the rise in titre was rapid, being approximately tenfold. This rise was followed by an abrupt fall to levels equal to those found at the commencement of the experiment. A second rise began at about 22 hours and was more sustained than the early rise. The maximal titre was reached in about 44 hours and later the titre leveled off or actually decreased slightly, this decrease being due presumably to the titration of less susceptible eggs which were the only ones to survive for 60 or more hours.

TABLE II

50 Per Cent Endpoints of Infectivity

Titration I		Titration II	
Time from inoculation to testing of yolk sac	Result	Time from inoculation to testing of yolk sac	Result
hrs.		*hrs.*	
0	$10^{-5.29}$	0	$10^{-5.17}$
4	$10^{-5.36}$	3	$10^{-5.33}$
12	$10^{-5.44}$	6	$10^{-5.55}$
15	$10^{-6.16}$	10	$10^{-5.5}$
18	$10^{-5.43}$	13	$10^{-6.11}$
21	$10^{-5.11}$	16	$10^{-6.43}$
24	$10^{-6.0}$	19	$10^{-5.45}$
28	$10^{-7.09}$	22	$10^{-5.57}$
36	$10^{-7.71}$	25	$10^{-6.0}$
39	$10^{-8.17}$	Titration III	
42	$10^{-8.6}$		
45	$10^{-8.81}$	36	$10^{-7.77}$
63	$10^{-8.36}$	42	$10^{-7.62}$
69	$10^{-8.19}$	48	$10^{-8.59}$
		60	$10^{-9.21}$
		70	$10^{-9.25}$

Some indication of the morphological changes which accompanied these changes in titre are given in Chart 1. These studies of morphology were, as has been pointed out above, carried out both by smear and by section. Up to the first 6 hours occasional virus bodies, elementary bodies, or others, could be seen in the smear or more rarely in sections. From their staining reactions and morphological characters it was clear that these bodies represented virus of the original inoculum. The period during which such bodies were found was always followed by a short period in which no virus could be observed in either section or smear, and this in turn was succeeded by the initial stages of the developmental cycle of the virus. These initial stages were always observed first in section and later in smear and, for the sake of convenience, the changes in the former will be described first. The picture of a cyclical de-

velopment which is given is open to the objection that it is obtained by the study of static preparations and must therefore be to some degree guesswork. However, it may be emphasized that the picture is a composite one which has been obtained by the examination of several hundred yolk sacs in which tissue the virus is very plentiful and extremely easy to see. Moreover the relationship of time to the stage of the development of the virus has been so precisely worked out that it is now possible to foretell with accuracy the picture which will be found in eggs opened at any given time after receiving a given number of infective units of virus.

Following the disappearance of the originally inoculated virus, new virus bodies, which might or might not be surrounded by halos, appeared in the yolk cells, where they lay close to the walls, at about 10 to 12 hours after original inoculation. Although in every instance the original inoculum consisted almost entirely of elementary bodies which showed after staining by the usual methods an average diameter of 400 $m\mu$, the initial virus bodies seen in the yolk cells had a diameter of about 1μ or more than twice that of the original virus particles. Since these initial bodies when first seen were very scanty and consisted of single bodies or at most pairs, it seems probable that the earlier stages of the cycle were overlooked on account of the small size of the original infecting units. These appeared to make use of the first 10 to 12 hours to increase in size until they were readily visible (Fig. 1). These initial bodies divided much like cocci to form pairs (Fig. 2), tetrads, and small groups (Fig. 3). By the time that these small groups (as in Fig. 3) had begun to appear, definite structural arrangements might be seen. The virus bodies appeared to be arranged within a vesicle with a limiting membrane which was more clearly defined in some instances than in others. The virus bodies might or might not be arranged close to the surface of this membrane and were already seen to be embedded in a thin matrix. Differential stains such as Noble's stain (31) showed that all of these virus bodies apparent at this time stained green with methylene green as did the thin matrix. No virus which had retained the basic fuchsin and therefore stained red appeared until later. Within a short time of the appearance of these small groups one began to see, besides an augmentation in number of these initial bodies, a further definite increase in size (Fig. 4). Thus by 16 hours bodies of 2μ in diameter were to be found and by 18 to 20 hours bodies 4μ in diameter were apparent. The matrix in which the bodies were embedded had become denser. By this time, moreover, definite structural differentiation might be seen within these larger bodies. With eosin-methylene blue stain, or Giemsa, some of the bodies of between 2 and 4μ were seen to be vacuolated (Fig. 5) while with Noble's stain at 18 hours a few of the larger green-staining masses were seen to contain one or more smaller muddy brown bodies which were undoubtedly bodies retaining the basic fuchsin seen through the dense green-staining surrounding material. By 20 hours definite bright red elementary bodies within large green bodies or plaques, or lying free were to be seen. This is shown in Fig. 6 in which a body about 3.5μ in diameter contains, besides a small vacuole, two small red bodies about 600 to 800 $m\mu$ in diameter embedded in the homogeneous dense green capsule. The plaques, already mentioned above, seemed to be produced by a steady augmentation in size of the larger bodies and only rarely, if at all, by the fusion of two or more bodies.

Several such plaques might appear in one vesicle (Figs. 7 and 8). They might reach a diameter of 4.5μ without showing any definite vacuolation when stained with eosin-methylene blue (Fig. 9) but most of the bodies of 3μ or greater were markedly vacuolated (Figs. 8, 10, and 11). Individual plaques might reach a diameter of at least 7μ (Fig. 8). In most cases with eosin-methylene blue nothing could be seen in the vacuoles of the large bodies or plaques but occasionally, as in Fig. 8, one might see quite definite little bodies of the size of elementary bodies in the vacuoles, and with the Noble stain many of the plaques were found to contain few or many red bodies of all sizes (Fig. 12). However, after the examination of many preparations one is forced to the conclusion that the plaques when they become large and show vacuoles are very fragile and in most cases lose their content of elementary bodies during the handling necessary in the production of the fixed sections. In Fig. 11 it would appear that the central plaque, which is obviously disintegrating, has liberated large numbers of elementary bodies which in this case are still to be seen closely surrounding it.

The changes seen in the first 18 to 20 hours may be summarized as follows: The first bodies were seen in the yolk cells at 10 to 12 hours after inoculation and at this time were more than twice as large as elementary bodies. During the next 6 hours these bodies increased in number (to 40 or more) and size (up to 4μ) and came to occupy small vesicles in which they were embedded in a thin matrix. Between 18 and 20 hours the bodies increased still more in size and formed plaques which might be 7μ in diameter. These latter showed vacuoles and with certain stains were seen to contain small granules of elementary body size. By their disintegration elementary bodies were set free and the first cycle might be said to have been completed. While therefore in many cases the first cycle might continue for 30 hours or longer, in some cases it took only 18 hours, this being the shortest period that we have noted. It must be emphasized that all the diameters given are those of fixed and stained virus bodies.

The liberated elementary bodies in many cases escaped from the affected cell and entered new cells to initiate new cycles of the same type as that described above.

The first appearance of this new cycle, as heralded by single or paired initial bodies close to the walls of otherwise normal yolk cells, was at 28 to 30 hours, *i.e.* 10 to 12 hours after the "reinoculation" with new elementary bodies, thus confirming the 10 to 12 hour "silent" period noted in the first cycle. If, however, they did not enter new undamaged cells, the history of the elementary bodies seemed to be different. New small vesicles were formed in which the elementary bodies divided for the most part as such so that the vesicles came to contain enormous numbers of elementary bodies embedded in a thin matrix. Just below the limiting membrane bodies of 1μ or greater might occur but they were less numerous than the elementary bodies. Fig. 13 to 16 show the development of these vesicles. From small size (Fig. 13) they came to occupy the whole cell (Fig. 14). Later many cells are filled (Fig. 15) and finally, at the point of death of the embryo, nearly all the cells are so affected (Fig. 16). It is

difficult to distinguish individual elementary bodies in the central mass but larger bodies may be seen close to the periphery of the vesicles.

As has been pointed out above, virus appeared in the smears or impressions from 3 to 6 hours later than it did in the sections. This was undoubtedly due to the fact that in smears the virus was derived by liberation from yolk cells damaged usually by the progress of infection and more rarely by the manipulation used in making the preparations. In the early hours virus was too scanty in the cells, and cell damage was so slight, that no bodies appeared in the smears. Once virus did appear in these preparations, however, it went through the same cycle as that seen in the sections.

At about 14 hours one began to see single virus bodies and even small groups similar to that shown in Fig. 17. The similarity of this to the group shown in Fig. 3 (in a section) is immediately apparent. Slightly later the groups were larger and by 20 hours were seen to contain bodies of the size of elementary bodies (Figs. 18 and 19). With Macchiavello stain the majority of these bodies stained blue up to 18 hours but even at 13 hours red, large bodies were seen. Later the proportion of red bodies to blue increased but at all times some blue bodies, even of the size of elementary bodies, were to be seen. Intact plaques were not seen in smears and any plaques were rare, but every now and then plaques in the process of disintegration were seen (Fig. 20). Scattered fragments of matrix were seen in the early stages, *i.e.* at 13 hours, but later larger masses of matrix in the process of disintegration were to be seen (Figs. 21 to 23). In Giemsa stains virus bodies could be seen in these masses, albeit with difficulty, but with Macchiavello stain red or blue virus bodies stood out clearly against the pale blue of the matrix (Fig. 23). In much later stages, *i.e.* at 48 hours, elementary bodies predominated but larger bodies were still to be seen. The so called dumbbell forms described for psittacosis were seen and appeared to be forms undergoing division and separation (Fig. 24). At the time of death elementary bodies formed by far the majority of forms seen (Fig. 25). In some smears, yolk cells containing large numbers of elementary bodies were seen (Fig. 26), or others in which the masses of elementary bodies remained intact although dislodged from the yolk cell (Fig. 27). Frequently one saw monocytes containing virus bodies (Fig. 28) but this would appear to represent only phagocytosis of the virus particles. A photograph (Fig. 29) of colonies of staphylococci growing in the yolk cells is inserted for comparison of size.

With Noble's or Macchiavello's stain the majority of elementary bodies forming a new inoculum retained the basic fuchsin and stained bright red. However, as has been pointed out above, the initial bodies were invariably green with Noble's stain, and no red bodies were seen until about 18 hours when brownish red bodies (or red bodies apparently partly obscured in green capsular material) were seen. With Macchiavello's stain red initial bodies might appear in smears, albeit rarely, as early as 13 hours. Eighteen hours is the time of the first appearance of elementary bodies and it might appear from this that the elementary bodies were the essential virus bodies, that they always retain the basic fuchsin when exposed to it, but that in the early stages of the cycle they were concealed in a dense capsule which stained with methylene green (or blue) so that they could not be seen until the capsule became

larger and less dense. That this simple explanation will not suffice, however, is shown by the fact that at no stage were all of the elementary bodies red (and this is particularly true with Macchiavello's stain), and furthermore that red bodies might occur of at least 2μ in diameter, that is, as large or larger than the initial bodies. It is the opinion of the authors that the initial bodies are furnished with a capsular material of their own manufacture (as opposed to the matrix which appears to be derived from the cell) and that in the early stage both virus and capsule stain with methylene green or blue. Later, due to metabolic changes, much of the virus retains the basic fuchsin while the capsule continues to stain green or blue. This faculty of the virus to stain either with basic fuchsin or with methylene blue in Macchiavello's stain is shown by

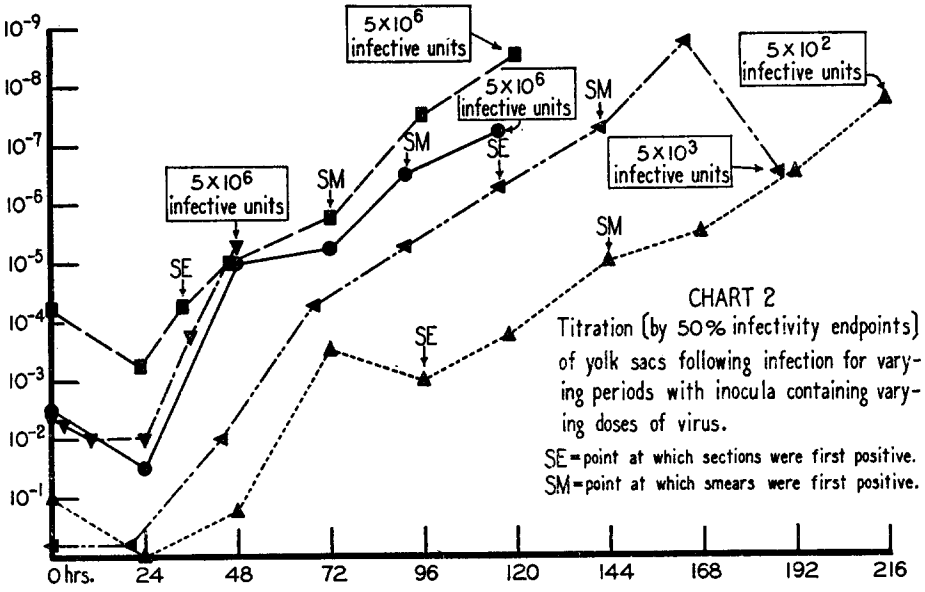

CHART 2

Titration (by 50% infectivity endpoints) of yolk sacs following infection for varying periods with inocula containing varying doses of virus.

SE = point at which sections were first positive.
SM = point at which smears were first positive.

those bacteria that we have studied. The metabolic changes responsible are not understood. The nature of either capsules or matrix has not been determined; it is not glycogen and in this respect the virus resembles that of psittacosis (32) and not that of trachoma (33).

When one correlates the changes in titre with the morphological appearances the following facts emerge. During the "silent" period of 10 to 12 hours during which no virus is observed in the yolk cells, only a very slight rise in titre occurs. Following the appearance of initial bodies, and coincident with the visual evidence of multiplication, the titre rises abruptly until about 16 or 18 hours. Then, at the time when elementary bodies first appear in section and larger numbers of virus bodies appear in the smear, the titre drops abruptly and may take 9 to 12 hours to regain its former level. It appears

probable that the appearance of elementary bodies is related to the rupture of many yolk cells and the discharge of virus into the yolk where most of it is lost in titrations of the yolk sacs. This loss into the yolk obscures, for several hours, the continued increase within the yolk cells, but this period is finally succeeded by another and more prolonged one in which the titre again rises steeply.

When smaller inocula are used as the initiating dose the results obtained are less spectacular and informative. It is very difficult to follow the results as closely as can be done during the short period from inoculation to death which follows the giving of a large inoculum. Two features which are usual in the titration curves obtained by the use of smaller inocula (Chart 2) merit attention. The first occurs in the first 24 hours and is a drop in titre below that obtained immediately following inoculation. It would appear to be due to a loss of infectivity on the part of many of the inoculated virus bodies. The second, which occurs at varying times from 48 to 96 hours after inoculation, depending on the size of the original inoculum, is a break in the steep rise in the titration curve, or even a decrease in titre, seemingly corresponding to the break occurring in the curve following the large inocula (Chart 1) which has been shown to occur at the conclusion of a developmental cycle and the consequent liberation of elementary bodies into the yolk.

DISCUSSION

Developmental cycles of equal or less complexity have been described in connection with other viruses. Thus Thygeson found larger initial bodies 0.3 to 0.8μ in diameter and smaller elementary bodies at a later stage in both inclusion blennorrhea (34) and trachoma (35, 36). As in lymphogranuloma venereum, division forms were frequent; in the vesicles or large colonies of elementary bodies the larger forms tended to lie at the periphery; and the larger bodies stained blue while the elementary bodies stained reddish blue with Giemsa. Thygeson noted the resemblance to the picture seen in psittacosis but pointed out (36) that the matrix surrounding the virus bodies in trachoma and inclusion blennorrhea could be shown to contain glycogen (33), which the matrix in psittacosis does not. We ourselves have not been able to demonstrate glycogen in the matrix in lymphogranuloma venereum.

Less resemblance to that of the agent of lymphogranuloma venereum is seen in the developmental cycle of vaccinia described by Bland and Robinow (37) but certain features of this latter cycle are reminiscent of the former.

When however attention is turned to the life cycle of the virus of psittacosis it is found that the resemblance between this and that described above for lymphogranuloma venereum is very striking.

Bedson and Bland were the first to draw attention to different developmental forms in psittacosis (32). Observing the spleens of infected mice they described, besides

the elementary bodies which stained deep purple with Giemsa, larger bodies of 1μ or more in diameter which might be circular or oval, appeared in pairs indicating division, and stained light blue with Giemsa. In addition, moreover, there were homogeneous plaques several μ in diameter of material staining with Giemsa like the virus bodies, which apparently divided to give rise to the larger 1 μ bodies. These in turn divided until the stage of elementary bodies was again reached, the whole cycle from elementary body back to the same stage taking 48 to 72 hours. In later papers (38, 39) the sequence of events was studied more carefully. It was found to be regular and completion of the cycle occupied 48 hours. The infecting elementary bodies gave rise to larger 1μ bodies very soon after entering the cells. These larger bodies multiplied as such and formed colonies embedded in a matrix. Finally the large forms by subdivision gave rise to very large numbers of elementary bodies. No conclusion was reached as to whether the elementary bodies multiplied as such. When Bland and Canti (40) followed the actual process in tissue culture (of chick lung epithelium) they noted first, at 8 hours, round or oval 5 to 10μ plaques which increased in size. Although these plaques appeared at first to be homogeneous, actually they contained many bodies 1μ in diameter. Later still at 18 to 24 hours the matrix became less dense and forms intermediate in size between the 1μ bodies and elementary bodies could be seen and by 72 hours nothing but elementary bodies was seen, embedded in a very light matrix. When these colonies of elementary bodies became enormous they eventually burst and set free numerous elementary bodies. Like Gey and Bang with lymphogranuloma venereum (30) they noted that the elementary bodies of psittacosis which lay in a light matrix surrounded by a definite limiting membrane, exhibited violent brownian movement. No development occurred outside the cells. Levinthal (41) also described the developmental cycle in chick embryonic tissue culture. He noted two types of cycle: (*a*) as it occurs in normal cells with large forms and plaques exactly as described by Bedson and Bland, and (*b*) as it occurs in damaged cells in which case the elementary bodies as such divide to form enormous colonies (vesicles) of virus bodies. Levinthal also noted what he termed involution forms, *i.e.* forms like dumbbells or others which were pale and vacuolated. Yanamura and Meyer (31) more recently have again investigated the developmental cycle in psittacosis and have confirmed in the main the results of the earlier investigators. Particularly when one examines the illustrations given in the above papers on psittacosis is one struck by the marked similarity between the picture in this disease and that in lymphogranuloma venereum. All the developmental forms described for the one disease can be exactly duplicated in the other.

The finding of the close morphological similarity between the agents of lymphogranuloma venereum and psittacosis is of great interest in view of other resemblances which exist between these viruses. Thus Rake, Eaton, and Shaffer (12) have shown that antigenic and tropistic similarities exist between the two viruses and others which appear to belong to the same group, and Jones, Rake, and McKee (11) have drawn attention to the fact that the carrier state may be as frequent and persistent in lymphogranuloma venereum as it is in psittacosis.

Our own studies described above have confirmed most of the description of the cycle as given by Findlay and have filled in many details. However, we have at no time seen any evidence to lead us to believe that the plaques are formed by aggregation of large bodies, rather one has been able to follow their formation by steadily increasing size of individual bodies; and the evidence for direct multiplication of elementary bodies as such would seem to be very strong since, for example, pairs and short chains are of common occurrence. On the whole our interpretation of the different picture seen at different stages after infection resembles most closely that given by Levinthal (41) for the cycle of development of the virus of psittacosis. In addition to the morphological changes we have been able to demonstrate constant changes in infective titre in the affected tissue and to correlate such changes to the morphological picture.

Bedson (38) and Bedson and Bland (39) believed that the large bodies in psittacosis had less virulence for mice than had the elementary bodies and gave evidence in support of this claim. There is no reason to believe that in the case of lymphogranuloma venereum any such decreased virulence of large bodies for the embryo occurs and, in fact, a consideration of the facts points against it. Thus, as is shown in Chart 1 or in Table II, an original inoculum of 5×10^8 infective units gives a titre of approximately $10^{-5.2}$ on immediate titration of the yolk sac. It is known that the infective units in this original inoculum are almost all elementary bodies. They cannot be seen in the yolk cells in sections prepared immediately after inoculation nor is any virus seen until about 10 hours when the larger initial bodies appear. It seems more than probable that such initial bodies, which are always single, when first seen and proceed shortly to divide, are derived by increase in size of the original elementary bodies. And this is borne out by the fact that the infective titre of the yolk sac remains approximately unchanged during this early period. It certainly does not decrease as would be the case if these initial bodies had a virulence less than those of the elementary bodies from which they are derived.

SUMMARY

Making use of the fact that the cells of the yolk sac of the developing embryo are readily infected with the agent of lymphogranuloma venereum and that the virus bodies can be readily observed in these cells because of the structure of the latter, the development of this agent has been followed at short intervals. It has been found to go through a regular cycle of development similar to that described for psittacosis in the spleen and less fully for lymphogranuloma venereum in the brain of infected mice. The development as observed microscopically can be shown to run parallel to changes in the infective titre of the yolk sac as tested in other eggs.

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EXPLANATION OF PLATES

PLATE 9

FIG. 1. Single virus body. Eosin-methylene blue. ×1200.

FIG. 2. Pair of virus bodies. Eosin-methylene blue. ×1200.

FIG. 3. Early vesicle containing several virus bodies. Eosin-methylene blue. ×1200.

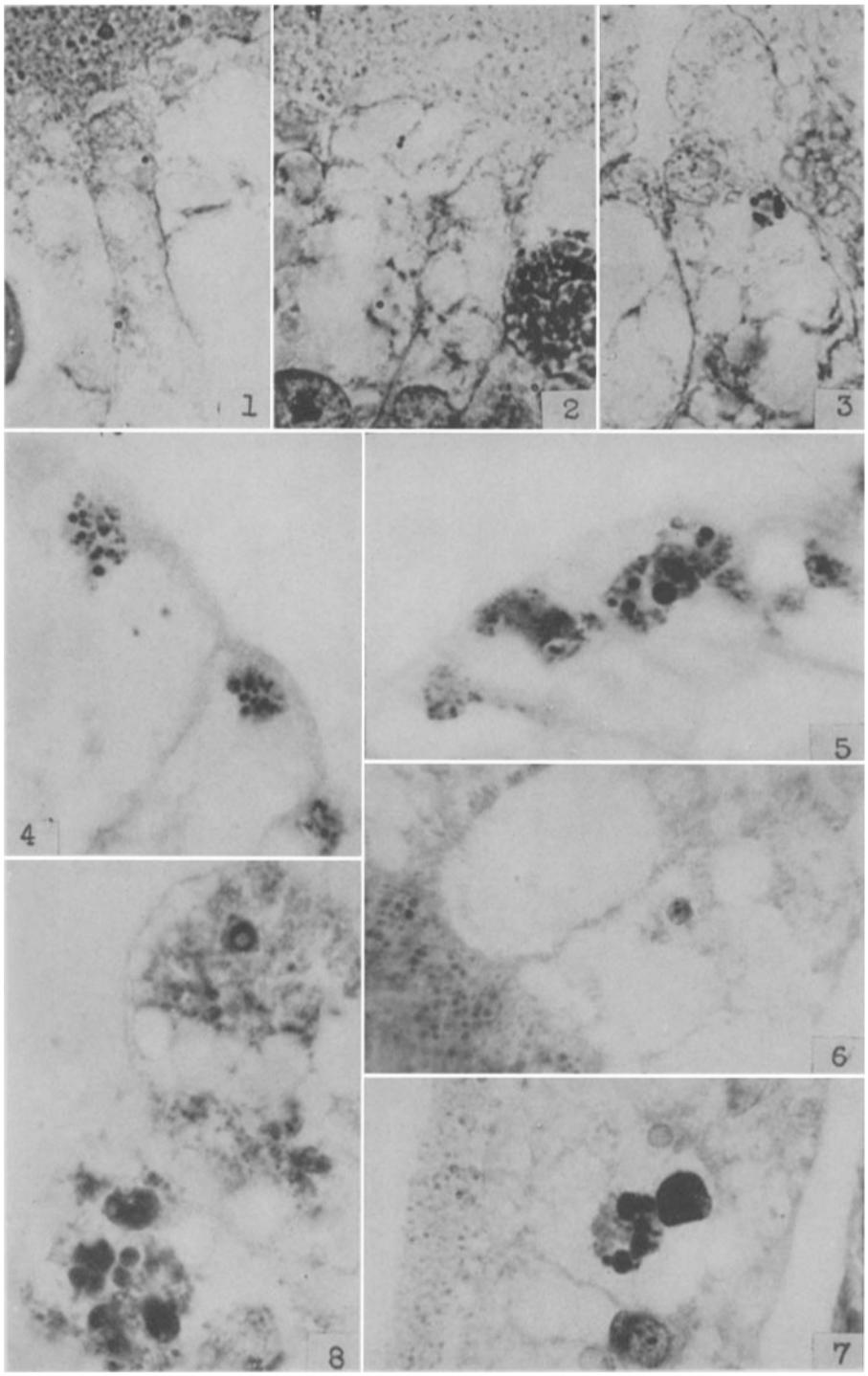
FIG. 4. Larger virus bodies and groups. Eosin-methylene blue. ×1200.

FIG. 5. Larger virus bodies, one at top vacuolated. Eosin-methylene blue. ×1200.

FIG. 6. Elementary bodies in plaque. Noble. ×1200.

FIG. 7. Group of plaques and large bodies. Eosin-methylene blue. ×1200.

FIG. 8. Multiple vacuolated plaques. Eosin-methylene blue. ×1200.



(Rake and Jones: Lymphogranuloma venereum. I)

PLATE 10

FIG. 9. Disintegrating and intact plaques. Eosin-methylene blue. × 1200.

FIG. 10. Vacuolated plaque. Eosin-methylene blue. × 1200.

FIG. 11. Disintegrating vacuolated plaque surrounded by elementary bodies. Noble. × 1200.

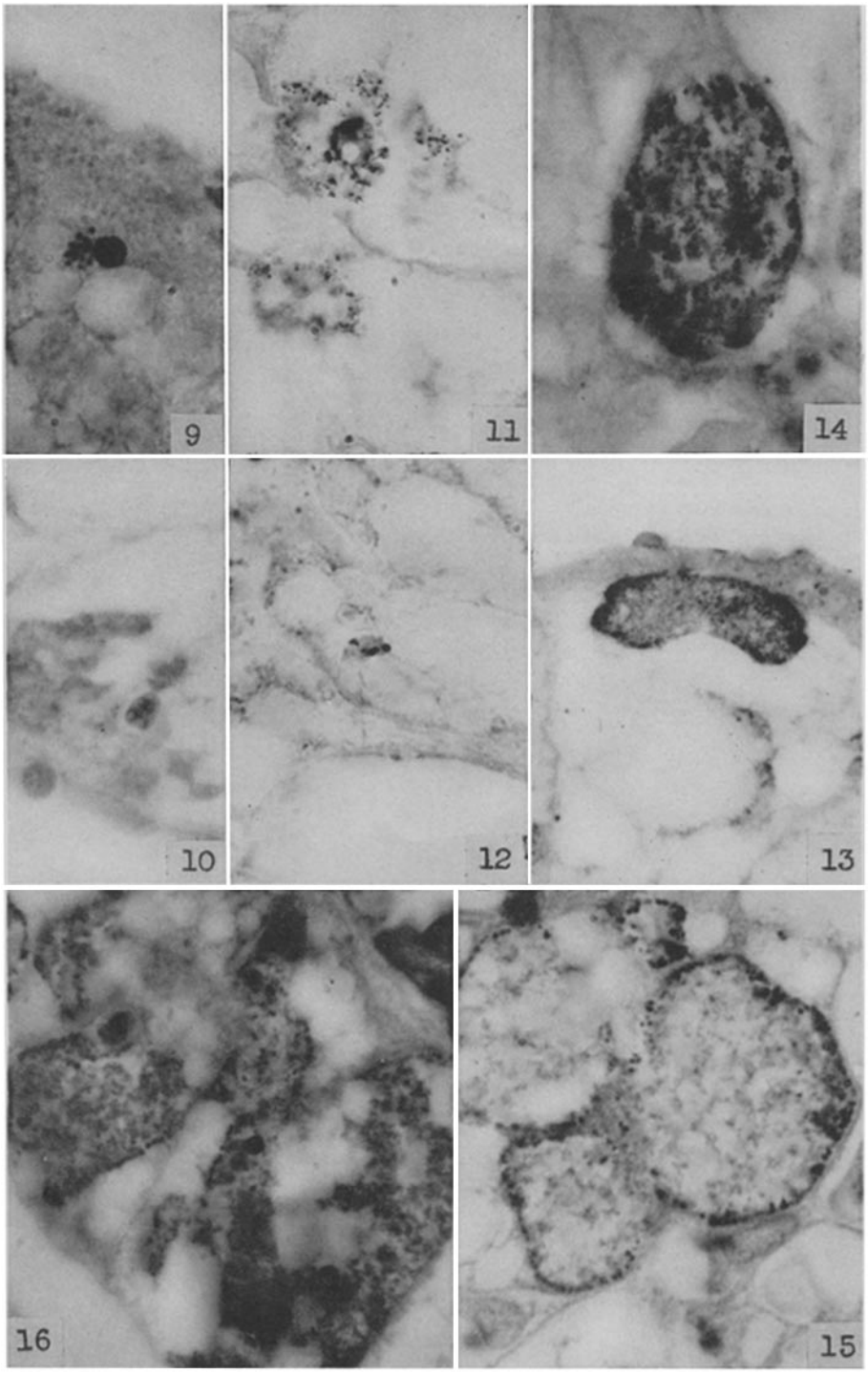
FIG. 12. Virus bodies in plaque. Noble. × 1200.

FIG. 13. Medium vesicle. Eosin-methylene blue. × 1200.

FIG. 14. Large vesicle. Eosin-methylene blue. × 1200.

FIG. 15. Three large vesicles. Eosin-methylene blue. × 1200.

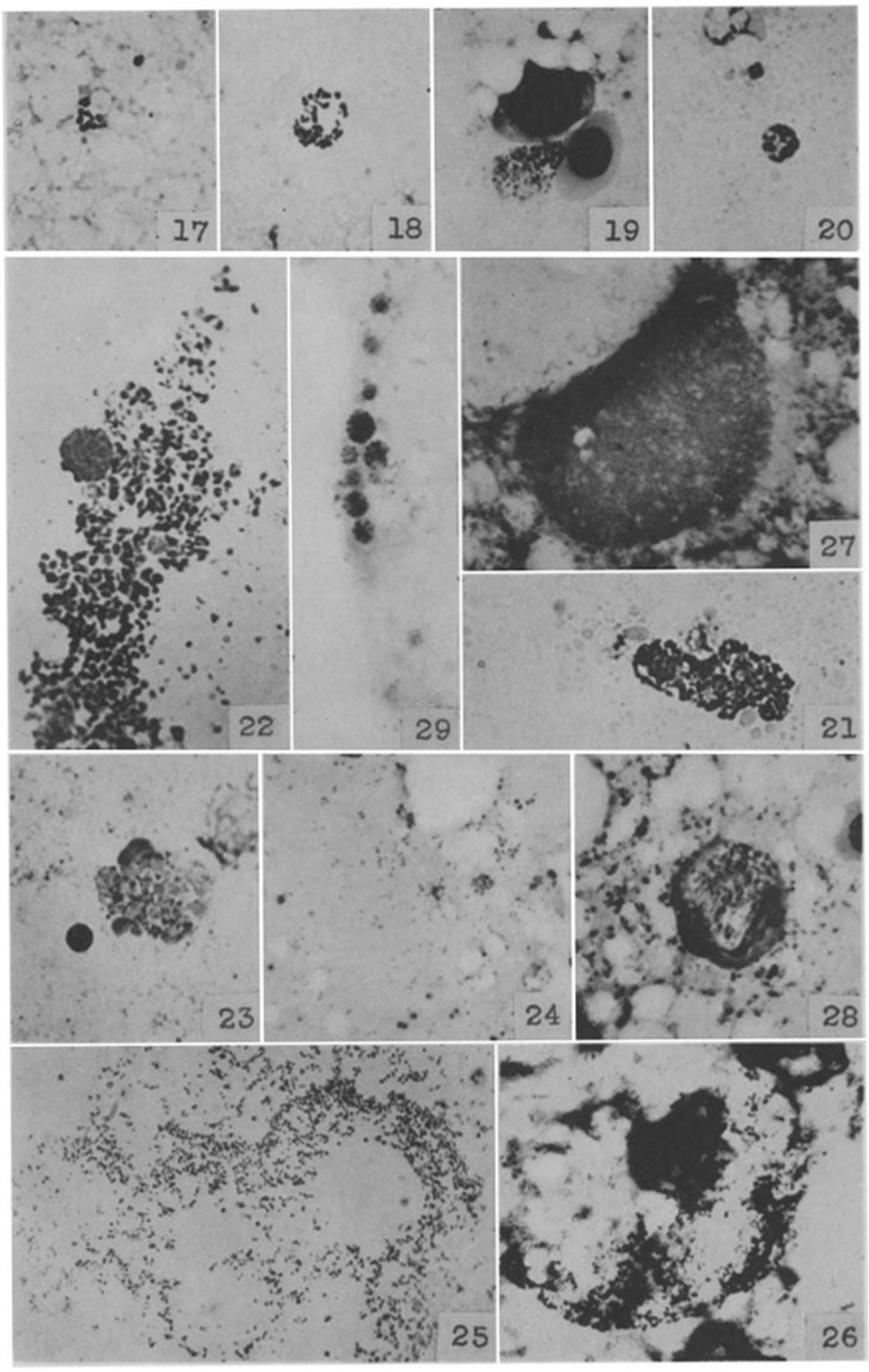
FIG. 16. All cells filled with virus. Eosin-methylene blue. × 1200.



(Rake and Jones: Lymphogranuloma venereum. I)

Plate 11

Fig. 17. Group of virus bodies in smear. Giemsa. × 1200.
Fig. 18. Large group of virus bodies in smear. Giemsa. × 1200.
Fig. 19. Group of elementary and larger virus bodies. Smear. Giemsa. × 1200.
Fig. 20. Disintegrating plaque. Smear. Giemsa. × 1200.
Fig. 21. Disintegrating matrix. Smear. Giemsa. × 1200.
Fig. 22. Disintegrating matrix. Smear. Giemsa. × 1200.
Fig. 23. Disintegrating matrix. Smear. Macchiavello. × 1200.
Fig. 24. Elementary and pair virus bodies. Smear. Giemsa. × 1200.
Fig. 25. Elementary bodies. Smear. Giemsa. × 1200.
Fig. 26. Elementary bodies in yolk cell. Smear. Giemsa. × 1200.
Fig. 27. Large mass of virus from yolk cell. Smear. Giemsa. × 1200.
Fig. 28. Phagocyted virus in monocyte. Smear. Giemsa. × 1200.
Fig. 29. Colonies of staphylococci in yolk cells. Eosin-methylene blue. × 1200.



(Rake and Jones: Lymphogranuloma venereum. I)