THE BACTERIOPHAGES OF *PSEUDOMONAS AERUGINOSA* FILTRATION MEASUREMENTS AND ELECTRON MICROSCOPY

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(With Plates 1–5 and 2 Figures in the Text)

A group of bacteriophages isolated from cultures of *Pseudomonas aeruginosa* as reported by Don and van den Ende (1950) has been selected for further study. Delbrück, Luria, Anderson, Cohen and others (see Delbrück's review, 1946) have already made detailed studies of the T group of coli phages and the susceptible host *Escherichia coli B*, and the results of their investigations have contributed greatly to our knowledge of the physical-chemical properties and the growth of bacterial viruses. The *Ps. aeruginosa* strains, most of which appear to be lysogenic, and their phages constitute a system in which the host-parasite relationship may be different from that of *coli B* and its phages, and their investigation may shed additional light on the nature and multiplication of viruses.

The first step in the systematic study of the *Ps. aeruginosa* (pyocyaneus phages) has been an attempt at classification on the basis of size and morphology. The evidence which is presented in this paper shows that although many of the phage strains isolated are probably variants of a single type there are, nevertheless, a number of distinct varieties of bacteriophage carried by these lysogenic hosts.

MATERIALS AND METHODS

Seventeen phages, arbitrarily numbered 1 to 17, were selected. They were all active on agar plate cultures of one or both of two variants (*Ps.* 2 and *Ps.* 3*b*1) of a single strain of *Ps. aeruginosa*, which appeared to be the most stable of the twenty-four variants of the red pigment producing strain L (Don and van den Ende, 1950). Two of the phages, nos. 4 and 8, were subsequently lost or discarded. Phages 1–3 were propagated on the variant *Ps.* 2 and the remainder on *Ps.* 3*b*1.

Transfers from single plaques of each phage were made into 1 ml. amounts of susceptible culture in the early logarithmic phase. After overnight incubation the culture was centrifuged and the supernatants titrated for phage. The single plaque isolation was repeated and the phage-containing supernatant fluids used to prepare larger stocks of the bacteriophages. The stock suspensions were titrated and stored in screw-capped bottles in a refrigerator at 4° C., under which conditions the titres were maintained satisfactorily for months.

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Titrations of phage

Most of the titrations were carried out by plating 0·1 ml. amounts of serial 100-fold dilutions of the phage with approximately 0·1 ml. of an overnight broth culture of the susceptible bacterium on well-dried nutrient agar (1 %) plates. The overnight culture always possessed a heavy surface pellicle unless it had been continually aerated or mechanically agitated during incubation. Before use in phage titrations the cultures were thoroughly shaken and left to stand for 1–2 hr., after which the upper layer of uniformly turbid bacterial suspension could be easily separated from the bottom layer containing coarse fragments of pellicle. The phages generally produced readily discernible plaques, but the exception, phage 17, yielded such minute plaques that to see and count them a hand-lens was necessary. The area covered by a single drop from a pipette calibrated to deliver 50 drops per ml. was suitable for counting through a lens, and hence for titrating phage 17 the '50 dropper' technique of Miles & Misra (1938) was used. Plaques were in all cases counted after overnight incubation of the plates at 37° C.

One-step growth experiments

A technique similar to that described by Delbrück & Luria (1942) was used. Actively growing cultures of the host organism, with an estimated density of 10^7 per ml., received a phage inoculum of such a size as to give a phage : bacteria ratio of approximately 1 : 1. After an adsorption time of 5 min. the mixture was rapidly diluted down in three steps to 10^{-5}. A sample from the 10^{-4} dilution was immediately chilled and centrifuged at 6000 r.p.m. for 3 min. Phage counts on the supernatant of this tube gave an estimate of the proportion of unadsorbed phage. From the experimental 10^{-5} dilution incubated at 37° C. 0·1 ml. samples were removed at 5–15 min. intervals for phage and bacterial counts. Air was bubbled through the culture tubes throughout the experiment to ensure uniformity of mixing and the absence of pellicle formation.

Filtration experiments

Freshly prepared 100 ml. batches of each phage were clarified by centrifugation or filtration through a Seitz clarifying pad and then filtered through gradocol membranes of an average pore diameter (A.P.D.) of 460–480 mμ. These stock filtrates were stored at 4° C. When required they were warmed to room temperature and 10 ml. amounts filtered under pressure (5–15 lb./sq. in.) through 150, 95 and 60 mμ membranes. The stocks and filtrates were titrated for phage content.

Preparation of phage concentrates

Bacteriophage lysates were prepared in 300–500 ml. amounts. In each case the bulk of the bacteria was removed by centrifugation and the supernatant clarified by filtration through a Seitz clarifying pad. The resulting clear phage suspension was concentrated by ultrafiltration through a reinforced collodion sac under a pressure of approximately 15–20 in. of mercury.

The sacs were prepared by pouring collodion (commercial 'Necol' 356/A diluted 1:2 with a mixture of ethyl alcohol and ether in equal parts) over selected Pyrex glass test-tubes (7 in. long × 1 in. diameter) slowly rotating (30 r.p.m.) about their long axis held horizontally. A small hole in the bottom of each tube facilitated the subsequent removal of the sacs. Two layers of collodion were poured over the tubes which were left rotating for a further period until the collodion had set in a uniform layer. The tube was then removed from the rotating spindle, allowed to dry in air for an additional 15 min., and then plunged into water. After soaking for 15–30 min. the collodion sac could be slid off the tube. Treatment in boiling water hardened the collodion membrane and greatly reduced the permeability. A sufficient reduction of bacterial contamination was achieved by storing the membranes for at least 12 hr. in 60–70% alcohol. Before use they were rinsed in sterile distilled water and a sterile rubber bung carrying a glass tube was tied into the open end of each sac with a strong rubber band. To give increased support to the membrane under pressure a 2 in. surgical bandage was carefully applied to the outside, paying particular attention that the end was well supported. Later it was found more satisfactory to incorporate the surgical bandage in the collodion membrane. After the first layer of collodion had been poured over the rotating glass tube a layer of gauze bandage was carefully applied to the whole surface. The second coat of collodion was poured slowly over the bandage allowing the collodion to soak thoroughly into the gauze. When gelation had taken place the tube was soaked in water and the reinforced sac was slid off the tube and stored in alcohol. No further support was required for such membranes which have been successfully used under pressures of 25 in. of mercury. Filtration was allowed to proceed, preferably at 0°, for 12–18 hr., which was the time usually required to reduce a volume of 300 ml. to 10 or 15 ml. The crude concentrate was then removed from the sac, taking care to wash down very thoroughly the inner surface of the sac where much of the phage was held. Bacteria were removed by centrifugation for 30 min. at 2500 r.p.m. A 5 ml. sample of each crude concentrate was purified further by differential centrifugation on an 'Ecco' high-speed centrifuge: a preliminary spin at 6000–7000 r.p.m. for 10 min. to remove bacteria and coarse debris followed by centrifugation at 10,000 r.p.m. for 3 hr. to deposit the phage. In the special type of conical glass centrifuge tube used the deposit was easily seen to consist of two distinct layers. The upper layer could be removed by gentle manipulation with a Pasteur pipette. In the first preparations the two layers were separately emulsified in buffered saline and titrated. The bottom layer proved to have the higher phage content and the upper fraction was in subsequent experiments discarded. The bottom layer of deposit was re-suspended in 1·0 ml. of buffered saline, a sample withdrawn for titration and to the remainder osmic acid was added to a final concentration of 0·02%. After 30 min. fixation the phage was again deposited by centrifugation at 10,000 r.p.m. for 1½ hr., the supernatant discarded and the deposit re-suspended in 0·5 ml. of distilled water. A further low-speed spin removed any coarse aggregates.

Preparation of specimens for electron microscopy

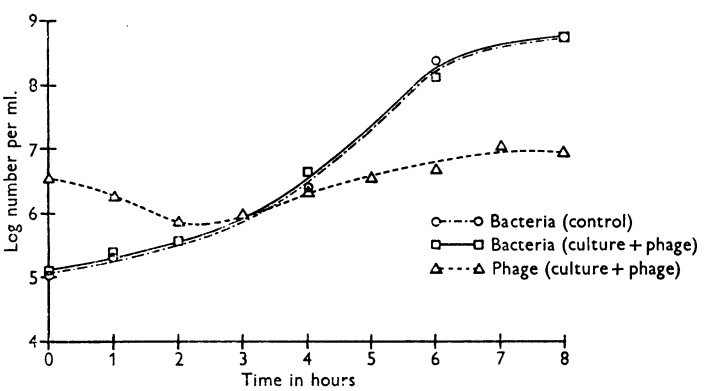
Suitable dilutions, in water, of the fixed, concentrated and partially purified phage preparations were placed as drops on thin films of collodion supported on a perforated platinum disk. The drops were allowed to dry over phosphorus pentoxide.

In a few experiments, unfixed phage was placed directly on the collodion film. After allowing 5–15 min. for phage to adhere to the film the excess fluid was removed, and a drop of 0·02 % osmic acid placed on each grid. After a 30 min. period of fixation the osmic acid was removed and the grids washed three times with distilled water, and then dried, as before, over phosphorus pentoxide.

RESULTS

Bacterial and bacteriophage multiplication

Actively growing cultures of *Ps.* 2 and *Ps.* 3*b* 1, infected with bacteriophages 1 and 5 respectively, showed no visible lysis and only slight or no reduction in the numbers of viable bacteria compared with control cultures without added phage. The results for *Ps.* 3*b* 1 and phage 5 are recorded in Text-fig. 1.

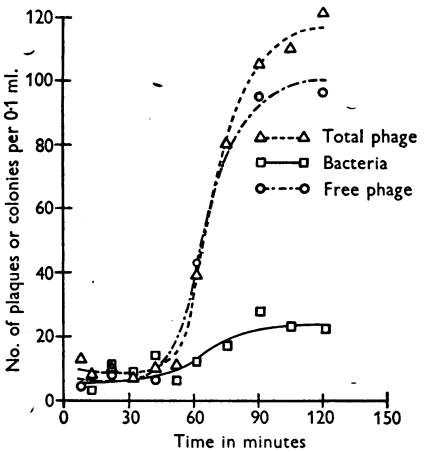


Text-fig. 1. Growth of *Ps. aeruginosa* strain L 3*b*1 alone and in the presence of phage 5.

One-step growth curves, however, for phages 1 and 12 showed the sudden increase in phage count generally associated with the sudden bursting of phage-infected bacteria. The burst occurred in each case after a latent period of approximately 45–50 min. There was in both cases a simultaneous increase in the number of bacteria (Text-fig. 2). In one experiment the amount of free phage was determined at intervals throughout the period of observation by plating supernatants from centrifuged samples from the experimental tube. Although the increase in both phage and bacteria occurred simultaneously the increase in phage was the greater, due mainly to free phage originating from those organisms that had actually lysed. The number of infective centres due to fixed phage also showed an increase equivalent to the increase in bacteria.

That bacteria can produce phage, though remaining viable, is suggested by the results of experiments in which subcultures were made from a culture growing in

the presence of phage. Single colonies from such subcultures were sampled into broth. The broth cultures after overnight incubation were tested for phage sensitivity as well as phage content. Sensitivity to phage was tested by plating 0·1 ml. amounts together with 0·1 ml. of a phage suspension containing 1000 phage particles per ml. Phage production was tested by titrating the supernatant fluids after centrifuging each culture. Cultures from such single colonies were always either fully susceptible to lysis by the phage, or, while apparently insusceptible when tested on a plate, produced in fluid culture a high concentration of the phage with which the original culture had been infected.



Text-fig. 2. One-step growth curve of phage 1 infecting *Ps. aeruginosa* strain L 2.

Filtration

The results of filtering the pyocyaneus phages through gradocol membranes of four grades are presented in the titration figures in Table 1. The phages studied may be divided into two groups: (1) those retained by a 95 mμ membrane, nos. 3, 10–14, 16 and 17; and (2) those that pass a 95 mμ membrane in reduced concentration but are completely retained by a 60 mμ membrane, nos. 1, 2, 5–7 and 15. In the absence of detailed filtration curves the orders of particle size of the phages in the two groups are assessed as 60–75 and 30–40 mμ respectively (see Elford, 1933).

Electron microscopy

The electron microscopic studies have been exploratory in nature. Highly active fractions of concentrates of phage filtrates have been examined and compared with fractions from cultures of the host organisms submitted to similar preparative treatment without addition of phage. While in some instances there were striking differences among the phage preparations, a common background of disk-like bodies and fibrils always accompanied those particles considered to be the phages since the frequency of their appearance in the field paralleled the activity of the preparation. Considerable light was thrown upon this general picture by the observations on control preparations from broth cultures of the two host strains of *Pseudomonas* grown without addition of phage. Electron micrographs revealed

the presence of small disk-shaped structures usually sharply outlined and sometimes umbilicated, ranging from 50 to 200 mμ in diameter, giving the impression of tiny collapsed cysts (these are illustrated in Pls. 1, 2 and Pl. 3, fig. 1). Such structures, though usually free, were seen attached to the outer surface of organisms which had not been removed by centrifugation of unfiltered material. In addition, the control preparations contained small spherical particles and filaments of irregular size as well as fragments of flagella. The host strains *Ps.* 2 and *Ps.* 3*b* 1 are known to be lysogenic and this fact obviously contributed to the intricacy of the problem.

Table 1. *Titres of phage filtrates through graded membranes*

	Porosity of membrane in mμ			
Phage	460	150 ($x = 140$)	95	60
1	$1{\cdot}5 \times 10^8$	$3{\cdot}7 \times 10^7$	$2{\cdot}3 \times 10^4$	0
2	$9{\cdot}9 \times 10^8$	$7{\cdot}1 \times 10^8$	7×10^3	0
3	1×10^8	8×10^6	0	0
5	$2{\cdot}5 \times 10^9$	9×10^8	3×10^4	0
6	$1{\cdot}5 \times 10^9$	8×10^8	5×10^5	0
7	$4{\cdot}8 \times 10^9$	$4{\cdot}7 \times 10^8$	$8{\cdot}7 \times 10^2$	0
10	$1{\cdot}7 \times 10^{10}$	$x 5{\cdot}4 \times 10^9$	0	0
11	$3{\cdot}6 \times 10^9$	$x 1{\cdot}1 \times 10^9$	0	0
12	$2{\cdot}0 \times 10^9$	$3{\cdot}1 \times 10^8$	0	0
13	$2{\cdot}5 \times 10^9$	$x 1{\cdot}6 \times 10^8$	0	0
14	$7{\cdot}0 \times 10^8$	$1{\cdot}9 \times 10^3$	0	0
15	$7{\cdot}4 \times 10^9$	$1{\cdot}6 \times 10^9$	$1{\cdot}4 \times 10^3$	0
16	$4{\cdot}2 \times 10^9$	$x 5 \times 10^5$	0	0
17	$4{\cdot}6 \times 10^9$	3×10^8	0	0

The figures are representative of two and in some instances three experiments with each grade of membrane.

Table 2. *Plaque appearances of pyocyaneus phages*

Phage	Plaque appearance
1, 2	Large haloed plaque 4–5 mm. in diameter. Central papilla is surrounded by trough which in transmitted light is opaque
3, 5, 6, 7, 9, 10, 15, 16	Pin-point to $\frac{1}{2}$ mm. shallow saucer-shaped pits with slightly irregular edges. Whole, except centre, of larger plaques opaque
11, 12	0·5–1 mm. shallow saucer-shaped pits with regular edges and sharply defined peripheral ring of opacity
13	1 mm. saucer-shaped pits with narrow shelf or halo at periphery. Ring-like opalescence over peripheral zone of pit
14	1 mm. superficial pits with clear-cut though slightly irregular and sometimes slightly raised edges. Floor of pits flat or with central papilla. Whole, except centre, opaque
17	Very tiny superficial pits best seen with lens. Whole faintly opalescent

Three of the phages, nos. 1, 2 and 12, in partially purified preparations appeared to be characterized by compact spherical particles 30–50 mμ in diameter (see Pl. 4, figs. 1 and 2). The remainder of the series exhibited a head and tail structure (see Pl. 4, figs. 3 and 4; Pl. 5, figs. 1–4). The heads of these phages showed considerable variation in diameter (60–100 mμ) and in their compactness and stability. In many instances they appeared as deflated spheres often irregular in outline, fragmented and separated from the tails. The tails also varied among different phages. Some appeared relatively stout and rigid, while others were more delicate. Their lengths ranged from 150 to 250 mμ and their widths 15–20 mμ.

A valuable method for studying the interaction of phages with the membranes of the host cell consists in mixing the phage with a suspension of the shells of the organism. Pl. 3, fig. 2, shows a micrograph of *Ps.* 2 shells and unbroken organisms to which phage 1 was added. The shells were prepared by rupturing the organisms by shaking with fine glass beads and then washing to remove the liberated cytoplasmic granules. Unfortunately, the number of phage particles adsorbed per organism in this instance was small, but here and there they may be seen adhering to the membrane surface.

DISCUSSION

Although a complete classification of the pyocyaneus phages is not yet possible, the evidence provided by the present studies of size, morphology and plaque-appearances has made it clear that there are several different types. The filtration experiments have indicated two broad groups in which the active particles are 60–75 and 30–40 mμ in diameter respectively. The electron microscope has revealed some of the phages as compact spherical particles 30–50 mμ in diameter, while the majority are tailed particles with heads 60–100 mμ in diameter and tails 150–250 mμ in length by 15–20 mμ in width. In some instances free tails were in great excess over heads and were the predominating morphological feature. A similar observation has been recorded for *Staphylococcus* 'K' bacteriophage (Smiles, Welch & Elford, 1948), and the occurrence of rod-like and filamentous forms in certain coli-phage preparations has been described independently by Herčik (1950) and by Wyckoff (1950). Recently, too, in a study of bacteriophage attacking *Erwinia carotovora*, Chapman, Hillier & Johnson (1951) have described the diverse array of particles, including rod-like and filamentous structures, resulting from the lysis of the organism. The real significance of the rod-like forms is not understood. Likewise, the cyst-like structures which we found to occur in abundance in our preparations of pyocyaneus phages 1 and 2, as well as in the control cultures, demand further investigation.

The pyocyaneus phages may be studied with advantage in attempts to elucidate how phage multiplies in lysogenic bacteria. Phage multiplication in lysogenic strains may yet depend on the existence of a small percentage of the bacterial population which is fully phage-susceptible, and, once infected, can undergo lysis with explosive release of bacteriophage. The one-step growth curve experiments reported in this paper show that such a mechanism does operate when lysogenic pyocyaneus strains are infected with certain phages. On the other hand, an in-

crease in the number of bacteria accompanies the increase of bacteriophage in such instances. The results of tests on single colonies isolated from the phage-infected culture suggest that the organisms may remain viable while tolerating continuing parasitism by phage. Our observations are in general agreement with the views of Lwoff, Siminovitch & Kveldgaard (1950), who have conducted comprehensive studies of phage multiplication in lysogenic strains of *Bacillus megatherium*.

SUMMARY

Preliminary observations in a systematic study of fifteen bacteriophages isolated from *Pseudomonas aeruginosa* are described. The evidence as to size, morphology and plaque appearance indicates that this group of phages comprises several distinct types. The electron microscope reveals some of the phages to be simple spheres, but the majority are tailed particles.

The importance of this group in the study of the phenomenon of lysogenicity is pointed out.

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REFERENCES

Chapman, G., Hillier, J. & Johnson, F. H. (1951). *J. Bact.* **61**, 261.
Delbrück, M. (1946). *Biol. Rev.* **21**, 30.
Delbrück, M. & Luria, S. E. (1942). *Arch. Biochem.* **1**, 111.
Don, P. A. & van den Ende, M. (1950). *J. Hyg., Camb.*, **48**, 196.
Elford, W. J. (1933). *Proc. Roy. Soc.* B, **112**, 384.
Herčik, F. (1950). *Experientia*, **6**, 64.
Lwoff, A., Siminovitch, L. & Kveldgaard, N. (1950). *Ann. Inst. Pasteur*, **79**, 815.
Miles, A. A. & Misra, S. S. (1938). *J. Hyg., Camb.*, **38**, 732.
Smiles, J., Welch, F. V. & Elford, W. J. (1948). *J. gen. Microbiol.* **2**, 220.
Wyckoff, R. W. G. (1950). *Experientia*, **6**, 66.

EXPLANATION OF PLATES 1–5

Plate 1

Fig. 1. *Ps. aeruginosa* 2: showing flagella and a few disk-like bodies.

Plate 2

Fig. 1. *Ps. aeruginosa* 3*b*1: showing flagella and disk-like bodies.

Plate 3

Fig. 1. *Ps. aeruginosa* 2: concentrated culture fluid containing filaments, disk-like bodies and other particles.
Fig. 2. *Ps. aeruginosa* 2: shells and some intact organisms + phage 1.

PLATE 4

Fig. 1. Pyocyaneus phage 1: partially purified showing spherical phage particles and remnants of flagella.
Fig. 2. *Ps. aeruginosa* 2+phage 2: partially lysed organisms showing many disk-like bodies and some free spherical particles of phage.
Fig. 3. Pyocyaneus phage 3: partially purified preparation showing the head and tail structure.
Fig. 4. Pyocyaneus phage 5: phage with head and tail structure. Disk-like bodies also present.

PLATE 5

Fig. 1. Pyocyaneus phage 6: partially purified preparation showing the head and tail structures of phage.
Fig. 2. Pyocyaneus phage 10: partially purified preparation showing head and tail structure but with many free tails.
Fig. 3. Pyocyaneus phage 15: partially purified preparation showing head and tail structures and associated disk-like bodies.
Fig. 4. Pyocyaneus phage 16: head and tail structures but with large excess of tails.

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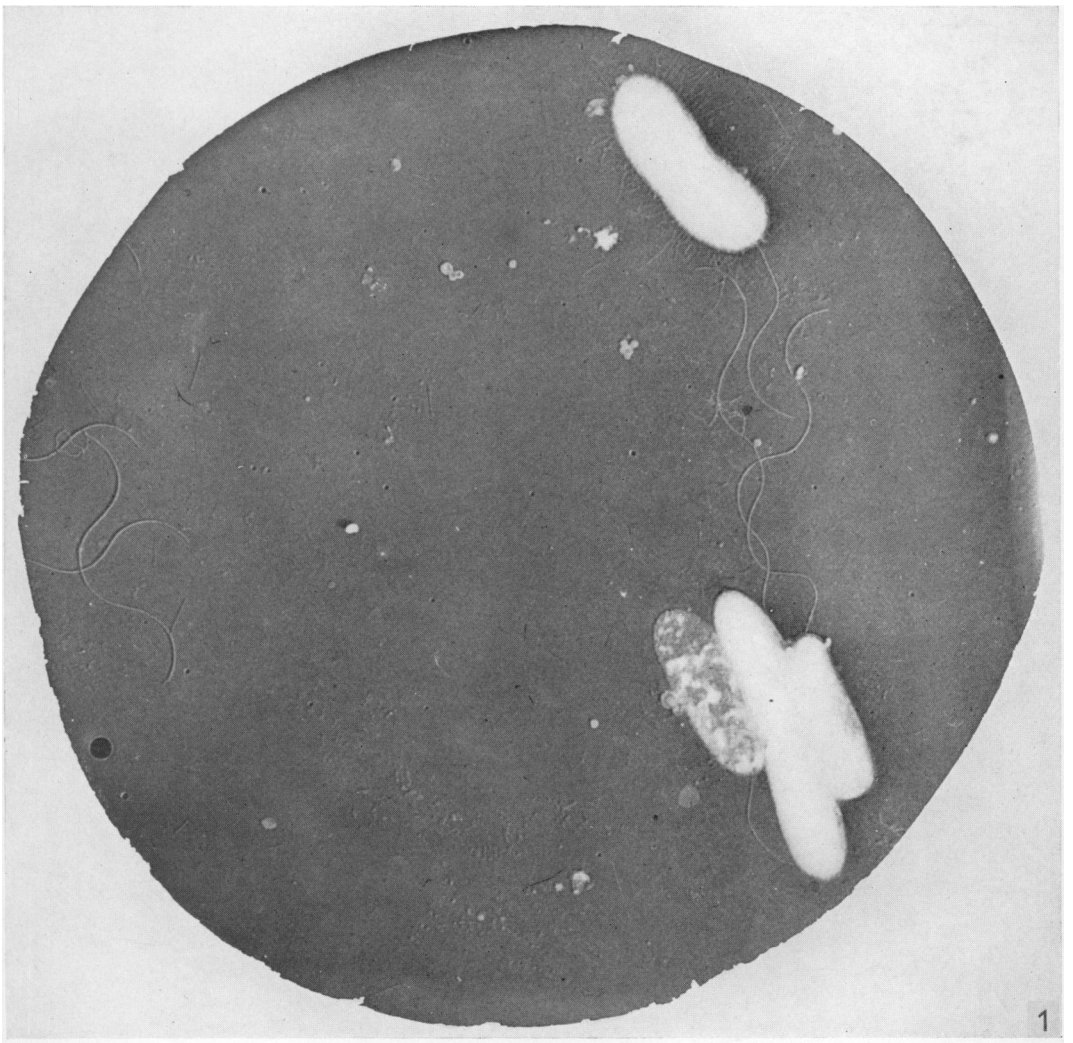
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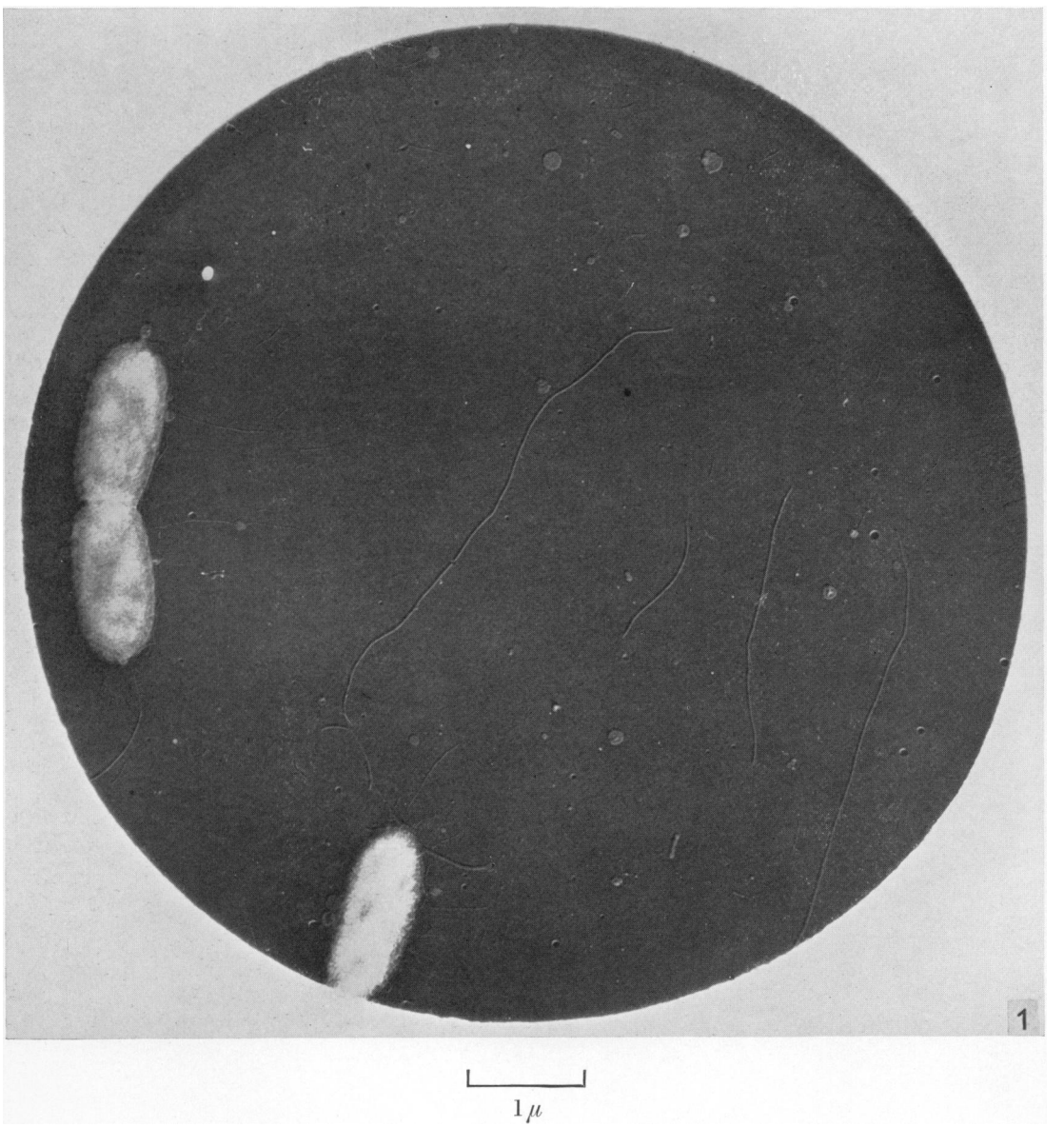
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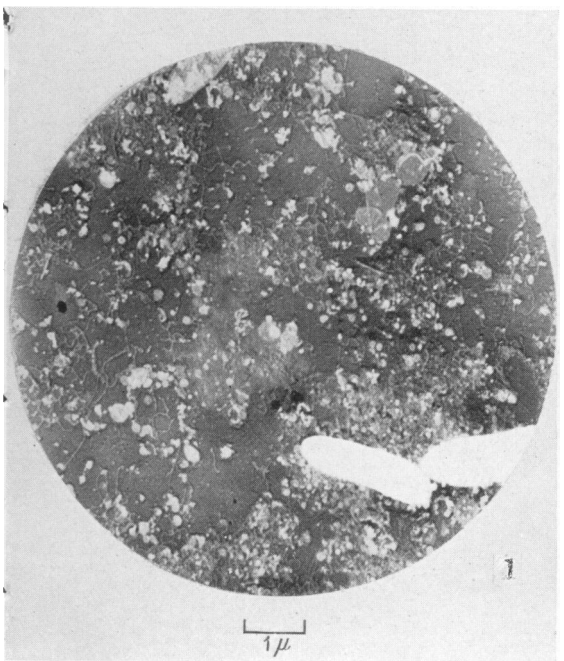
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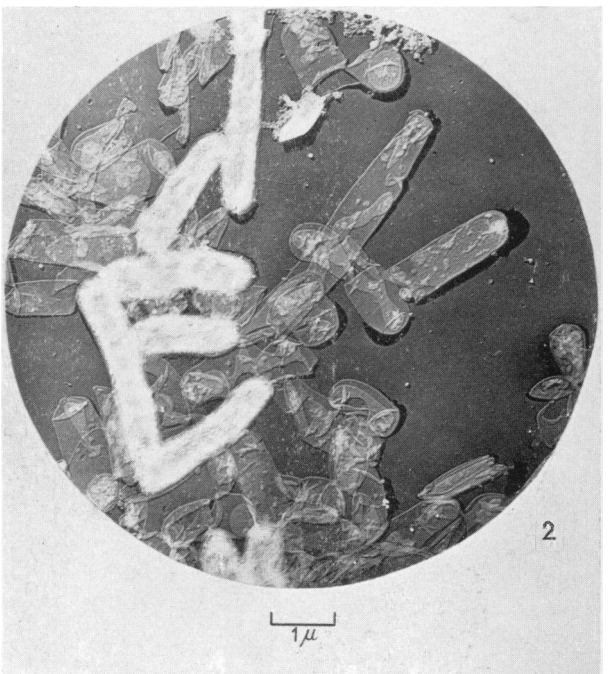
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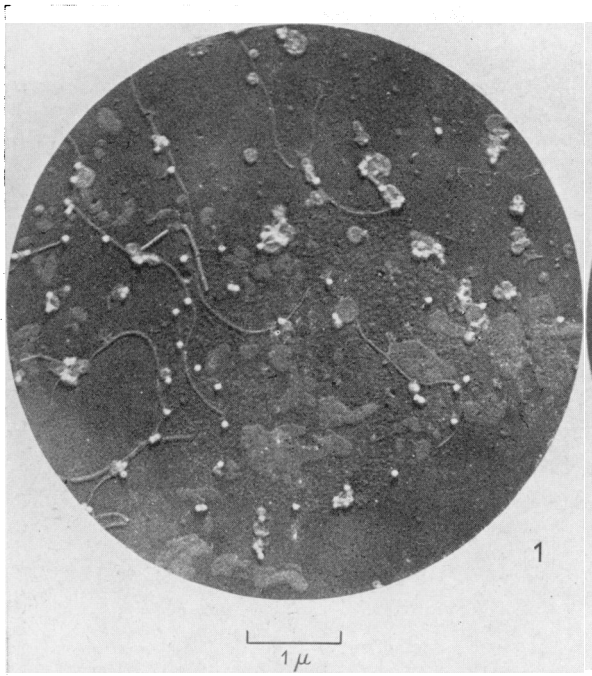
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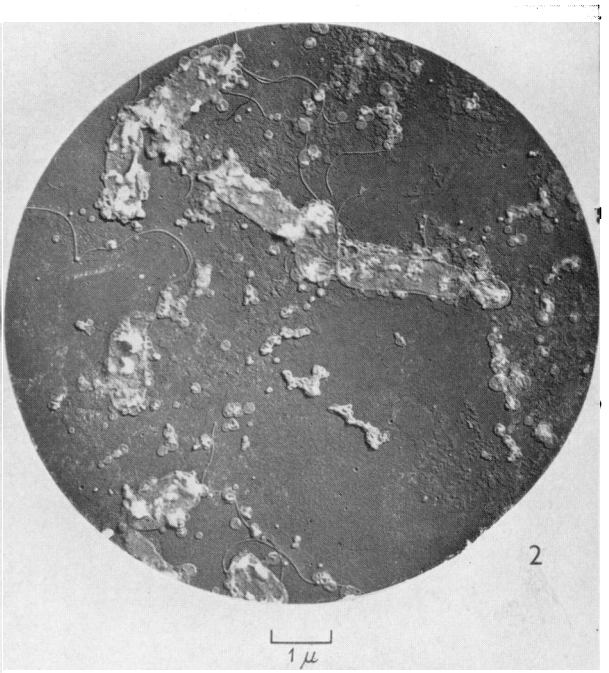
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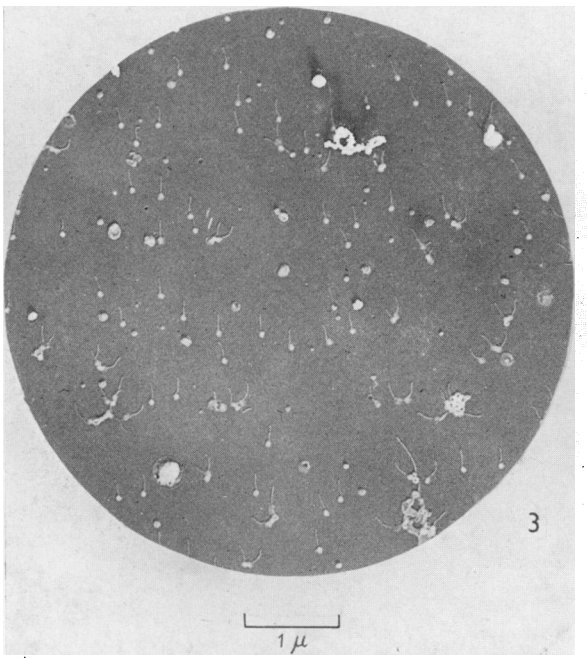
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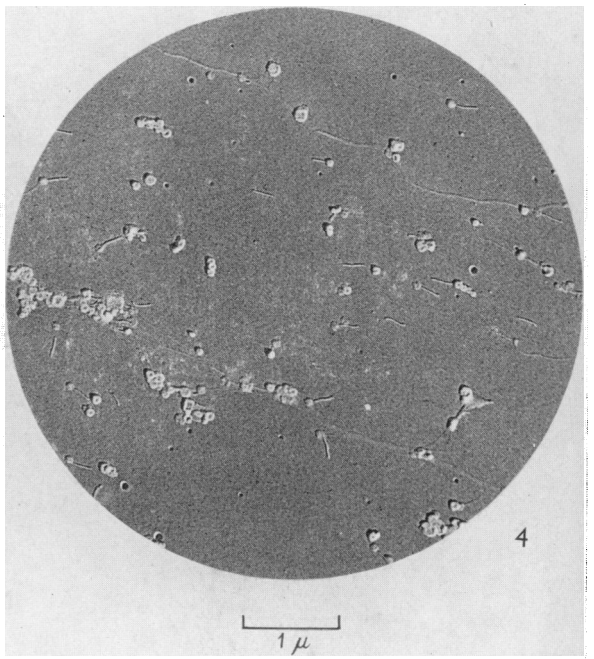
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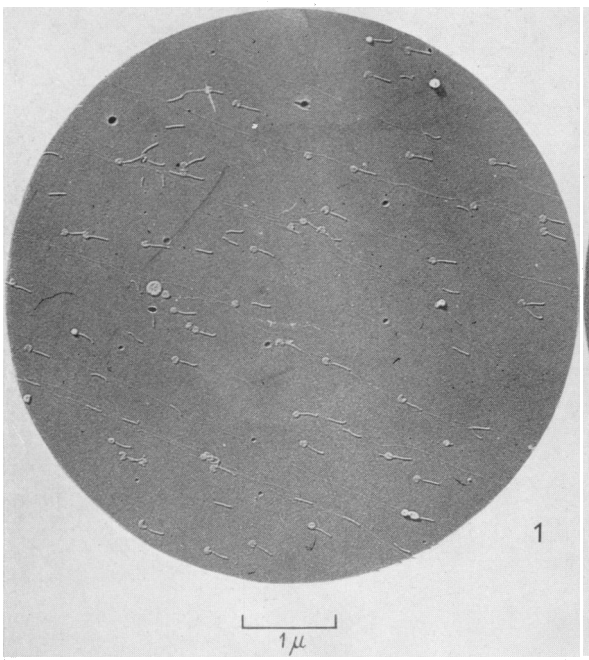
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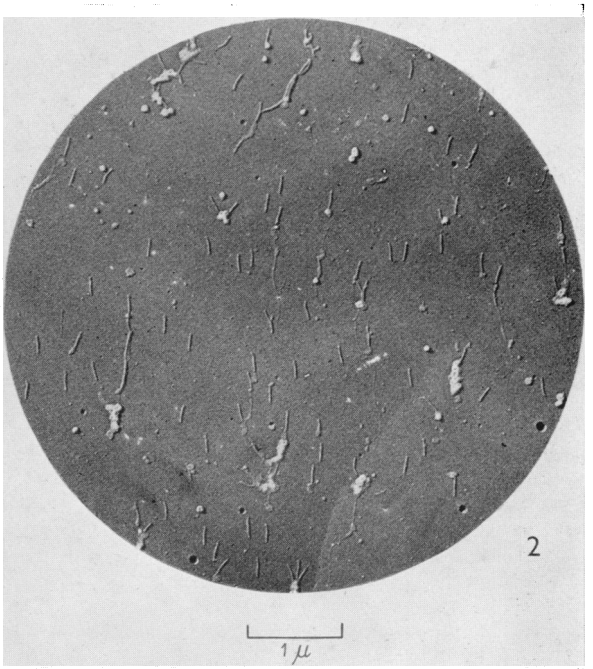
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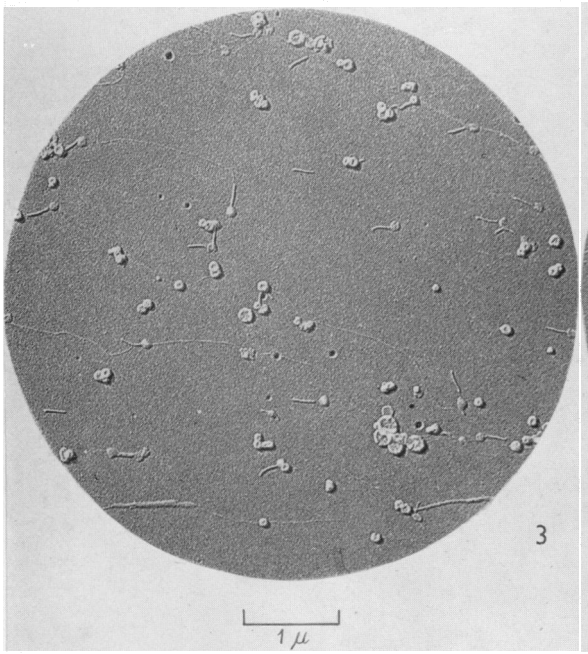
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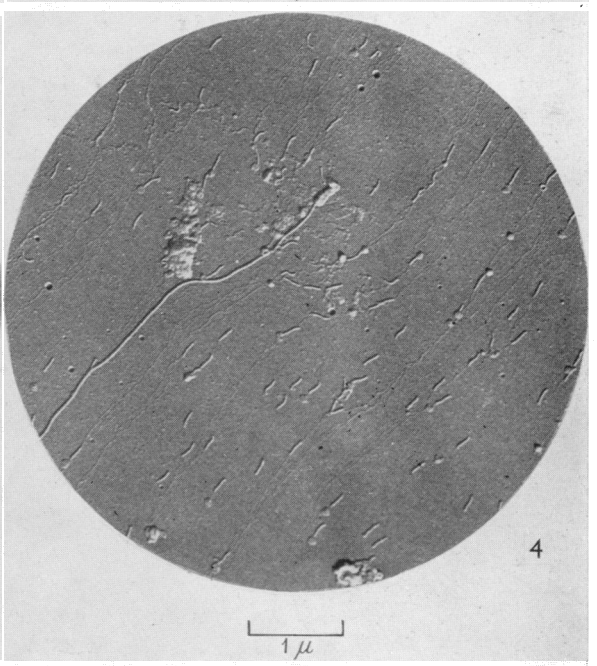
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