The Influence of Antibacterial Substances on the Interaction of Bacteria and Bacteriophages

2. Optical Studies of the Penicillin Effect

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SUMMARY: The ultra-violet light and electron microscopes were used to observe the effect of bacteriophage and penicillin on *Staphylococcus aureus*. Penicillin caused the organisms to swell to almost twice their normal size, and immediately before lysis they became less opaque to electrons and internal structure was evident. The triply segmented cocci seen in the ultra-violet light micrographs to be characteristic of penicillin-treated *Staph. aureus* appear to be due to the irregular arrest of the process of division, whereby a cell partly divides and then only one of the incipient daughter cells partly divides again.

The adsorption of the *Staph. K* phage to the surface of *Staph. aureus*, the latent period during which the coccus enlarges and the phage multiplies, and the lysis of the cell with liberation of phage particles were also recorded optically. Accelerated lysis of *Staph. aureus* by the combined action of phage and penicillin was not associated with any peculiarity in micrographic appearance, except that with high concentrations of penicillin the number of phage particles released on lysis was diminished.

During air- and freeze-drying the diameter of *Staph. aureus* contracts by 30–50%.

Electron micrographs of *Staph. K* bacteriophage, gold-shadowed and unshadowed, show a round head totally opaque to 50 kV. electrons, 50–60 m μ . in diameter, and a slender tail 200–250 m μ . in length.

The various phases of the combined action of penicillin and bacteriophage on *Staphylococcus aureus* (Elford, 1948) have been examined by means of the ultra-violet light and of electron microscopes. These two instruments have their own particular merits and shortcomings for an investigation of this kind and it may be well to consider them briefly.

The ultra-violet light microscope. In this instrument, employing wave-lengths 275 and 253 m μ ., the limit of resolution is decreased from 200 m μ . (that of the best visual microscope) to about 100 m μ . The specimen, dispersed in a medium transparent to ultra-violet light, is mounted between the quartz slide and cover glass, and examined either by directly transmitted ultra-violet light or the dark-ground method. The image presented by transmitted light is largely in terms of the specific absorption in the ultra-violet characterizing the various elementary structures in the object, whereas the dark-ground image is formed through the scattering of light. With biological specimens in particular, it is important to be able to study the object as nearly as possible in its natural state, i.e. in contact with its natural fluids or in a medium isotonic with them. Fixation treatment may alter structural details, and actual drying may completely transform the picture as the result of contraction, precipitation, crystallization and salting-out. As a means of minimizing these undesirable consequences the freeze-drying method suggests itself. Here it is hoped that by practically instantaneous freezing of the system and the subsequent

removal of water by evaporation from the ice phase there will be little, if any, contraction and distortion of the main elements of structure. The ultra-violet light microscope provides a means of assessing the magnitude of the distortions due to such fixing and drying treatments; specimens of the same material can be examined in the untreated condition, and after drying in various ways, the object can be remounted in a suitable medium such as castor oil or glycerol water. This possibility holds, of course, only for objects of relatively large sizes within the resolution limits of the ultra-violet light microscope. Clearly, therefore, the effects on such objects as bacteria and tissue cells can be analysed.

The electron microscope. The resolution limit of this instrument in its present stage of development is probably about $1\text{ }\mu\text{.}$, thus providing a means of bridging the gap between the lower limit of application of the ultra-violet light microscope and the upper limits of X-ray analysis. However, from the viewpoint of biological studies the electron microscope has the serious drawback that the specimens must be in the dry state. Attempts have been made to investigate moist specimens enclosed between collodion film (Abrams & McBain, 1944), but the technical difficulties are great and it would appear doubtful whether the system would remain sufficiently stable for reliable examination. Some of the possible changes occurring on drying have already been indicated and it will be evident that in evaluating the results of electron microscopy these factors must be borne in mind. The treatment of the object before, and during, the actual mounting for electron microscope study should always be recorded in detail, otherwise there can be no reliable interpretation of the images photographed.

EXPERIMENTAL

Materials. The Oxford 'H' strain of *Staph. aureus* (abbreviation, Staph. O) and Staph. K bacteriophage (abbreviation ϕ) were used throughout, in papain broth, Hartley's broth and Fildes's defined medium. The penicillin was a pure sodium salt of penicillin G having an activity of 1600 international units (u.)/mg. For further details see Elford (1948).

Ultra-violet light microscope. The Beck-Barnard microscope (Barnard & Welch, 1936; Elford, 1938) was used with the condensed cadmium spark as the light source for the wave-length $275\text{ m}\mu$. The highest power Zeiss Quartz monochromatic lenses gave a resolution of $100\text{ m}\mu$. Photographs were taken at an initial magnification 1100.

Electron microscope. The R.C.A. model 'B' electron microscope was used, with no limiting aperture in the objective pole piece. The energy of the electron beam was 50 kV. throughout.

Specimen preparation. Specimens for examination in the electron microscope were mounted on thin films of collodion supported on a disk of fine nickel gauze (mesh $50\text{ }\mu$.). These films were prepared by allowing one drop of a 2% solution of 'necoloidine' in amyl acetate to spread on the surface of water. Samples from the experimental systems were centrifuged and the deposit resuspended in water. This treatment was repeated and a drop of the resulting

aqueous suspension then placed on the collodion film. It was either dried in air over P_2O_5 or freeze-dried. For the latter, the disk with specimen was placed on a copper block and left standing in a freezing-bath of CO_2 -ethanol at -72° . The copper block with the frozen specimen was then transferred to a glass container in the vacuum line of a freeze-drying unit. The rate of evaporation was sufficient to keep the preparation frozen throughout the drying process, but the temperature was not kept at -72° . For study with the ultra-violet light microscope, specimens on quartz slides were similarly treated, and the dried organisms mounted in castor oil.

Experiments have been carried out by one of us (J. S.) to find out if it was possible to incorporate micro-organisms in a collodion film before dehydration by spreading 1 % collodion in amyl acetate directly on the moist specimen, and, if successful, whether distortion of the organisms and shrinkage in directions parallel to the plane of the film could be prevented. It was found that *Proteus vulgaris* and *Chromobacterium prodigiosum* could be mounted in this way, and that the resulting electron micrographs differed greatly from those obtained by the usual method of mounting described above. When air-dried on the surface of the film the organisms were totally opaque, or nearly so, to 50 kV. electrons, but when picked up from the surface of moist nutrient agar, or from a freshly prepared impression on glass, the organism yielded micrographs showing opaque internal structures in a relatively transparent cytoplasm. It was evident from the smooth outline of the organisms that serious distortion had been prevented and from the transparency of the cytoplasm that shrinkage accompanied by increase in mass density could not have taken place. Unfortunately, similar experiments with *Staph. aureus* were not successful.

RESULTS

Untreated staphylococci. Ultra-violet light micrographs of untreated Staph. O from cultures in the logarithmic phase of growth are shown in Pl. 1, fig. 1. The structure of the cell as it divides by binary fission is clearly seen. In contrast, the image of freeze-dried Staph. O in the electron microscope (Pl. 2, fig. 6), though indicating the shape and size, reveals nothing of the internal structure owing to the uniform opacity to 50 kV. electrons. A typical analysis of the size distribution in a normal Staph. O culture, from ultra-violet light micrographs, is given in Table 1, C; 80 % of the organisms have sizes in the range 0.75 – 0.90μ . Measurements from electron micrographs of freeze-dried Staph. O yielded the corresponding figure 0.6 – 0.7μ .

Staphylococci treated with penicillin. Penicillin in known concentration was allowed to act for a given period on Staph. O growing logarithmically in broth or synthetic medium and samples withdrawn for examination. In the presence of penicillin at 37° the staphylococci swell to almost twice their normal size and after prolonged contact are finally lysed. Typical analyses of size distribution are given in Table 1. Whereas in the early stages of penicillin action the electron microscope shows only the swelling of the organism (Pl. 2, fig. 7), the preparation being wholly opaque, the ultra-violet light microscope, on the

other hand, does reveal internal structure (Pl. 1, figs. 2 and 3). The process of binary fission is seen to be anomalous when penicillin is present. Most of the enlarged cells appear to have developed to the point when fission should occur and then stopped, and there is often a noticeable difference in ultra-violet light absorption by the two halves of the cell. Also, many of the cells show three segments (Pl. 1, fig. 3), suggesting that growth has proceeded to the point of

Table 1. *Frequency distribution of the diameters of Staph. O treated in different ways, measured on ultra-violet light micrographs*

System	Diameter (μ .)										No. cocci measured
	0.30	0.45	0.60	0.75	0.90	1.05	1.20	1.35	1.50	1.65	
A. Untreated Staph. O, freeze-dried	16	25	54	4	1	—	—	—	—	—	256
B. Staph. O + penicillin*, 1 hr. at 37°, freeze-dried	—	1	23	52	24	—	—	—	—	—	250
C. Untreated Staph. O, papain broth culture	—	—	5	25	55	12	3	—	—	—	325
D. Staph. O + penicillin*, 1 hr. at 37°, papain broth culture	—	—	—	—	16	25	51	8	—	—	239
E. Staph. O + penicillin*, 3 hr. at 37°, papain broth culture	—	—	—	—	—	1	46	29	20	4	140

* 0.1 u./ml.

division, and one of the daughter cells, without being detached, has developed to the point of mitosis and no further. These observations support the view held by many workers that penicillin prevents normal division of the organism. Following prolonged action of penicillin the staphylococci become transparent to 50 kV. electrons and before disintegration internal structure can be seen with the electron microscope. Certain elements, presumably nuclear, retain their opacity and the cytoplasm contains fine granules which are liberated when the cell membrane disintegrates. Similar granules can also be discerned in the ultra-violet light micrographs (Pl. 1, fig. 3). These points are well illustrated in electron micrographs of material deposited by centrifugation from a culture lysed by penicillin (Pl. 3, fig. 8). These show cells in various stages of lysis, remnants of cells and yet other cells apparently highly resistant to the antibiotic.

The influence of drying on staphylococci. Cultures of Staph. O in broth and defined medium were examined by ultra-violet light micrography, both as wet specimens, and remounted in castor oil after air- and freeze-drying (Pl. 1, figs. 1 and 4). The size distribution is given in Table 1, A and C. In an untreated culture of Staph. O. 80 % of the organisms have sizes within the ranges 0.75–0.9 μ ., whereas the corresponding figure after freeze-drying treatment is 0.45–0.60 μ . Similar analyses of cultures containing 0.1 u./ml. penicillin (Pl. 1, figs. 2, 3 and 5; and Table 1, B and D) indicate that in the wet condition 75 % fall within the size range 1.05–1.20 μ ., while after freeze-drying the size range decreased to 0.6–0.75 μ . The contraction due to drying is therefore 30–50 %.

Measurements on electron micrographs of freeze-dried organisms (*c.* 20 measured in each case) indicated the following peaks in the distribution of sizes. Untreated staphylococci, 0.6–0.7 μ .; staphylococci treated with 0.1 u./ml. penicillin for 1 hr. at 37°, 0.8–1.1 μ .; and after 3 hr. treatment with penicillin

1-1.25 μ . These figures are slightly greater than those determined in ultra-violet light micrographs. It may well be that the surface with which the organism is in contact during drying in some measure affects the final size. A small error in calibration may also be involved. In view of these facts the two methods may be said to yield results in fair agreement.

Staphylococci infected with bacteriophage. Young cultures of Staph. O at 37° were inoculated with concentrations of Staph. K phage sufficient to produce multiple infection. Electron micrographs of the phage adsorbed on to staphylococci are shown in Pl. 3, fig. 9. When infection has lasted for about half an hour the cell, enlarged relative to normal size, ruptures and an increased number of phage particles is released. No clear-cut evidence was obtained as to whether the phage multiplies at the surface of the cell or within the cytoplasm. In some instances the phage appeared to be congregated around the periphery of the cell, while in others the particles were located nearer to the 'nucleus'.

The elementary phage particles. Preparations of phage from filtrates of lysed cultures of Staph. O in synthetic medium, concentrated and washed with water in the air-driven centrifuge, were examined in the electron microscope. Staph. K phage comprises a round head opaque at 50 kV. electrons and a relatively long slender tail. Pl. 4, fig. 11, is a micrograph of a specimen treated with 0.02 M-CaCl₂ to enhance the contrast (Taylor, Sharp, Beard, Beard, Dingle & Feller, 1943). Measurements of the head indicated a diameter 50-60 m μ . and a tail length of 200-250 m μ . Pl. 4, fig. 12, shows Staph. K phage gold-shadowed by the method of Williams & Wyckoff (1945). The beaded appearance of the tail we consider to be an artefact due to the character of the metallic deposit. Pl. 4, fig. 10, is very interesting; a concentrated Staph. K phage preparation, washed several times in distilled water, has aggregated on the collodion with heads uniformly arranged as a layer over the film surface. The heads do not appear to be in contact, but are accompanied by an apparently fibrillar material. Further studies on concentrated preparations of this phage are in progress.

Staphylococci attacked by phage and penicillin. The general sequence of events following a combined attack by phage and penicillin on staphylococci differed little from that following infection by phage alone. After initial adsorption the majority of the particles was attached to the surfaces of the organisms and at the end of a definite latent period the enlarged cell disintegrated, liberating elementary phage particles. Lysis appeared to consist of a general dissolution of the cell wall and not the bursting of a bag that otherwise remained intact. After lysis, certain elements, possibly nuclear, that are opaque to electrons are frequently seen, similar to those remaining when penicillin or phage acts alone. The micrographs (Pl. 4, fig. 13) show cells soon after lysis by phage + 1 u./ml. penicillin. The escaping phage particles are clearly depicted, and appear to have been liberated in most cases from the peripheral regions of the cell, often as though propelled outwards as a wave but sometimes as a mass following the sudden dissolution of the cell. There is a wide variation in the number of particles escaping per cell, but the average figure for concentrations of penicillin not exceeding 0.1 u./ml. is much the same as for phage alone.

DISCUSSION

Our optical studies amply confirmed the conclusion drawn from direct analyses of the action, combined and singly, of phage and penicillin on staphylococci. Gardner (1940), Smith & Hay (1942) and Fisher (1946) have shown in microscopical studies that the organisms swell considerably in size under the action of penicillin. Weiss (1943) and recently Shanahan, Eisenstark & Tanner (1947) have used the electron microscope to study the effect of penicillin on various organisms. Their observation of the initial swelling of the organisms, culminating in the lysis of the cell, accords with our own. Our work with ultra-violet light micrography, in addition to confirming the general swelling of the organism in the presence of penicillin, throws further light on the stage at which growth and development of the organism are arrested; it is clear from the internal structure that the cell is unable to divide normally. It appears that one daughter cell, without being detached, is capable of further growth, again to the point of division. This leaves the enlarged cell divided into three segments, a picture characteristic of penicillin-treated staphylococci. Nothing similar was observed in any of our untreated staphylococci in the logarithmic growth phase. It would not be at all surprising, however, if such structures were occasionally encountered in untreated senescent cultures. In the period between the initial swelling of the cell in the presence of penicillin and lysis, the cell became increasingly transparent to 50 kV. electrons, so that an internal structure could be seen in electron micrographs, as, for example, in the disintegrating pair of cells (Pl. 3, fig. 8).

The electron microscope has been of great service in studying the process of infection of staphylococci by phage. It has shown the phage particles adsorbed to the cell surface, the enlarged cell during the latent period and, finally, elementary phage particles escaping from the cell on lysis (cf. Ruska, 1942; Luria, Delbrück & Anderson, 1943; Edwards & Wyckoff, 1947). The evidence is still not conclusive as to precisely where the phage multiplies. The balance of evidence favours the peripheral regions of the cell. If this be indeed the case, then the 'depressor effect' described by Delbrück (1945), whereby excess of superficially adsorbed phage influences the multiplication of intracellular phage, is more understandable. Unfortunately, the size of the phage particle is just below the resolution limit of the ultra-violet light microscope. Nevertheless, by photographing 0.1 μ . optical sections through the cell, the presence of adsorbed phage particles could be detected on the outer surface of the cell, though nothing definite could be learned regarding the whereabouts of particles within the cell.

The pictures of the combined action of phage and penicillin were very similar to those of phage acting alone, except that with high concentrations of penicillin the number of phage particles escaping from the lysed cell was less.

Staph. K phage was shown by means of the electron microscope to possess a round head, opaque to 50 kV. electrons, and a relatively long slender tail. The diameter of the head in the dried specimens is 50–60 m μ ., a figure agreeing well with the size indicated by ultra-filtration and centrifugation data, 60 m μ .

and 70 m μ . respectively for the phage in its normal wet state (Elford, 1938). The presence of considerable excess of 'free tails' and the association of phage with masses of 'fibrillar' material, particularly in the early stages of purification, still leaves us in doubt as to whether the tail is an essential part of the phage's morphology. Further evidence is needed on this point.

The observations on the shrinkage and distortion of staphylococci during air- or freeze-drying are of importance in the interpretation of electron micrographs, particularly in estimations of size. The contraction of 30–50 % on drying indicated in ultra-violet light micrographs is of the order of magnitude found by us for a number of micro-organisms studied by this method. Bacillary forms were particularly liable to distortion. Clearly, in any instance, the magnitude of the shrinkage and distortion will depend upon the object under investigation—viz. the rheological properties of the cell wall and of the cytoplasm, and on the density and size distribution of internal structure. Wyckoff (1946) has pointed out the need for minimizing the distortion due to desiccation, and has described a freeze-drying procedure very similar to our own. An additional advantage of the freeze-drying method in our experience was the more even distribution of organisms on the collodion film. It appears that any tendency to aggregation on the film, as may happen during the slower air-drying process, is largely eliminated by freeze-drying.

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Fig. 1-5

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Figs. 6 and 7

Figs. 8 and 9

Figs. 10-13

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EXPLANATION OF PLATES

PLATE 1

Ultra-violet light micrographs of *Staphylococcus aureus*, Oxford strain. Wave-length = 275 m μ . Magnification $\times 3300$.

- Fig. 1. Staph. O 1 hr. culture in papain broth at 37°.
- Fig. 2. Staph. O culture in papain broth incubated for 1 hr. at 37° in presence of 0.1 u./ml. penicillin.
- Fig. 3. Staph. O culture in papain broth incubated for 8 hr. at 37° in presence of 0.1 u./ml. penicillin.
- Fig. 4. Staph. O culture in papain broth for 1 hr. at 37°, concentrated by spinning and washed in water; freeze-dried and remounted in castor oil.
- Fig. 5. Staph. O culture in papain broth incubated 1 hr. at 37° in presence of 0.1 u./ml. penicillin, concentrated and washed in water; freeze-dried and remounted in castor oil.

PLATE 2

Electron micrographs of *Staphylococcus aureus*, Oxford strain. Magnification $\times 15,000$.

- Fig. 6. Staph. O from 1 hr. papain broth culture at 37°. Spun down and washed in distilled water; the film air-dried over P₂O₅.
- Fig. 7. Staph. O from papain broth culture incubated 1 hr. in presence of 0.1 u./ml. penicillin at 37°. Spun down and washed in distilled water and the film freeze-dried.

PLATE 3

- Fig. 8. Electron micrographs of deposit remaining after the action of 0.1 u./ml. penicillin at 37° for 24 hr. on Staph. O. The lysed culture was centrifuged 15 min. at 10,000 r.p.m., the deposit washed twice in distilled water and the film freeze-dried. Magnification $\times 15,000$.
- Fig. 9. Electron micrographs of Staph. O and Staph. K phage (multiple infection, 10–20 phage particles/cell), showing phage adsorbed to surface of cell and the infected cell in different stages of disintegration. Magnification $\times 15,000$.

PLATE 4

Electron micrographs of Staph. K phage.

- Fig. 10. Phage washed several times in distilled water in high-speed centrifuge. Air-dried on collodion film. Magnification $\times 30,000$.
- Fig. 11. Staph. K phage from lysed Staph. O in defined medium, centrifuged down and resuspended in 0.02 M-CaCl₂, again centrifuged and the deposit resuspended in water. Air-dried on collodion film. Magnification $\times 45,000$.
- Fig. 12. Staph. K phage from lysed Staph. O in defined medium, centrifuged and resuspended in water. Air-dried on collodion film and shadowed with gold. Magnification $\times 45,000$.
- Fig. 13. Staph. O attacked by phage and penicillin. Staph. O, 5×10^8 /ml. in logarithmic growth phase in papain broth + Staph. K phage, 10^{10} /ml. + 1 u./ml. penicillin; incubated 45 min. at 37°, cells centrifuged and resuspended in water. Air-dried on collodion film. Cells in various stages of lysis and phage particles being liberated. Magnification $\times 30,000$.

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