RECENT ADVANCES
IN
BACTERIOLOGY

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PREFACE

ANY reader who recollects the first edition of this book will see at once that the present volume is entirely new: indeed only an odd phrase here and there survives from 1928. This transformation corresponds to the revolution in bacteriological thought and practice which has occurred in the last two decades. The emphasis has moved from the description and novelty of new phenomena on the borderline between the laboratory and clinical medicine, to what is doubtless from the bacteriologist's point of view a more specialized and more fundamental plane. In consequence, bacterial physiology and the significance of bacterial classification, rather than the impact of bacteria upon pathological processes has become a matter of increasing interest at the moment.

Such an evolution of the subject has, as far as the scope of this book is concerned, resulted in some inevitable contraction as specialization has increased, so that virus diseases, which formed a considerable part of the earlier editions, are no longer included; virology being now a special branch of biology in its own right.

It should be stated forthwith that this volume is essentially the work of Dr. J. D. MacLennan. Its revision, which he began in 1939, was interrupted by the War and subsequently by travel and his migration to America. In consequence close collaboration in its production has been impossible and the business of editing the book and seeing it through the press has fallen wholly upon its earlier author. This separation in time and place is also responsible for a degree of inequality between certain of the chapters, some having been on the stocks longer than others and so, to this extent, may not contain accounts of all the most recent work: this, however, is not entirely avoidable in a book of this kind, especially when it is the work of a single individual. Dr. Mary Barber has had more than a small finger in the pie, for she has not only read the proofs but has assisted very materially in the preparation of certain chapters having, in the absence of Dr. MacLennan from this country, revised and brought up to date Chapter XV and herself contributed the final chapter.

It is with gratitude that we make certain other acknowledgments. Miss D. F. Atkins, the Librarian to the Postgraduate

Medical School of London, has undertaken the extremely formidable task of checking the 17,000 odd references. For the electron microphotographs we are mainly indebted to the good offices of Dr. Stuart Mudd and the generosity of the Society of American Bacteriologists, through their Chairman Dr. Harry E. Morton. Dr. Ralph W. G. Wyckoff and Dr. R. C. Williams have been most generous in supplying us with many beautiful examples of their outstanding achievements in this field, so that it has been an embarrassment to select a limited number of representative examples. Equally, Dr. Frank H. Johnson has placed us in his debt by allowing us the use of his remarkable photographs. To these scientists, to Dr. C. Robinow, some of whose beautiful preparations appear as Figs. 6, 8, 12 and 13; to Dr. Georges Knaysi for Fig. 7; Dr. H. W. Hoper for Fig. 11; and Dr. S. D. Elek for Fig. 14, we would offer our grateful thanks. To the publishers of the *Proceedings of the Society of Experimental Biology and Medicine*; the *Journal of Bacteriology*; the *British Medical Journal*; Interscience Publishers Inc., the R.C.A. Laboratories, and the Harvard University Press we are indebted for the use of such illustrations as are individually acknowledged. At the end we would record that Messrs. J. & A. Churchill have helped us in many ways and throughout the long gestation of this book have been models of patience and courtesy.

J.H.D.

London, 1951.

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CHAPTER I

THE MORPHOLOGY OF MICRO-ORGANISMS

DESPITE its obviously fundamental importance in almost every branch of bacteriology, our knowledge of the finer structure of micro-organisms has remained meagre. Nor is this to be wondered at: so far as cytological investigations are concerned most bacteria are approaching the limits of optical resolution, while the Rickettsiæ and the smaller viruses are definitely beyond it. The result has been a mass of observations which have tended to depend for their existence more on the preconceptions of the investigator than on any demonstrable detail. Indeed the literature on the subject, prolix, contradictory and fantastic, often bears a striking resemblance to that on the problems of Mars and its canals; and for very similar reasons. It is true that in the last twenty-five years numerous attempts have been made to confirm or refute older theories on bacterial anatomy by the aid of more refined techniques—by dark field illumination (Pijper), by special staining methods (Knaysi, Robinow), by the use of ultra-violet light microscopy (Barnard), and by micro-dissection (Wâmoscher), but no striking advances were made, nor did any seem possible along these lines. Recently, however, a new method of investigation has been developed, which has possibilities in the field of bacterial cytology that are almost infinite. This new tool for research is the electron microscope.

The Electron Microscope

The effectiveness or otherwise of an ordinary direct vision microscope depends on its powers of resolution, that is its capacity to distinguish two closely adjacent points. This in turn is limited by the numerical aperture of the objective lens-system employed and by the wave-length of light; these two factors are related by

the well-known formula of Abbe: $d=\lambda/2n \sin A$, where d is the limit of resolution, n the refractive index of the medium through which the light from the condenser passes, λ the wave-length of the light or, since n Sin A is the numerical aperture (N.A.) $d=0.5\lambda/N.A.$ Since visible light is of relatively long wave-length (not less than about 500mμ) and lenses with a numerical aperture greater than 1.4 are not attainable in practice, it will be clear that a limit of resolution is reached with particles of a diameter of about 0.2μ. Of course a further improvement is possible by using light, such as ultra-violet light, which is of shorter wave-length, and by this method Barnard has been able to photograph fully resolved particles of 0.07μ. But further advance along these lines is prevented by the fact that the quartz lenses used are not transparent to light of still shorter wave-length.

In 1925 de Broglie published his theory of matter, which suggested (among other things) that moving electrons have a wave motion of their own; and three years later Thompson in this country and Davisson and Gerner in America produced observational proof of this. It was found, as de Broglie had predicted, that the electronic wave-length was extremely short compared with that of light, and was a function of the velocity of the electron —the higher its speed, the shorter being the wave-length. Previous to this, in 1926, Busch had shown that the paths of electrons in electro-static or electro-magnetic fields could be described by certain simple equations, well known in optic physics—in other words, electro-static or electro-magnetic fields, provided they were axially symmetrical, behaved as lenses, and would focus an electron beam in a predictable manner.

So far as the production of an electron microscope was concerned the fundamental discoveries had been made. Their significance, however, was not appreciated for several years, and it was not until shortly before the last war that instruments suitable for biological work were developed. Since then numerous technical improvements have been devised, which need not concern us here. [For theoretical consideration, see von Ardenne (1940) and Zworykin *et al.* (1945).]

The essential structure of the more commonly used electron microscope is shown diagrammatically in Fig. 1. From this it will be evident that the electron microscope is in many ways comparable to the ordinary light microscope—each has a source of

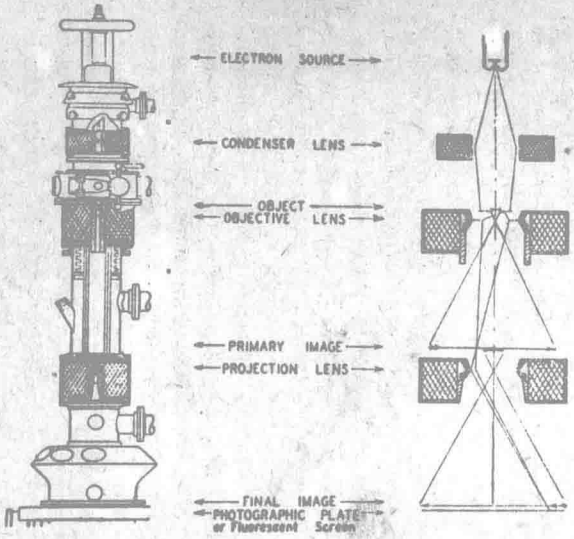
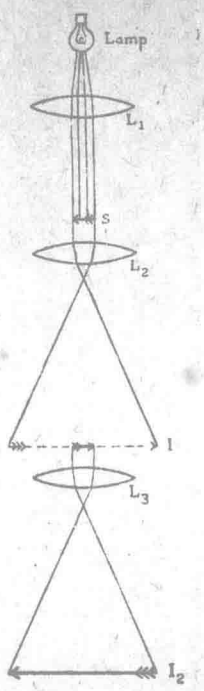
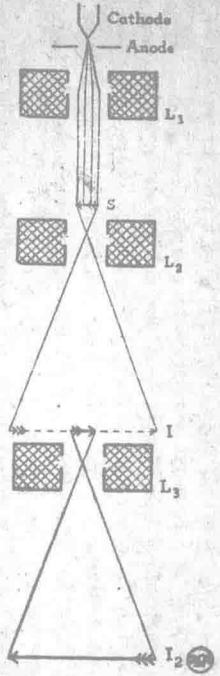


FIG. 1. The electron microscope. On the left is a scale drawing and on the right a schematic diagram of the instrument showing the arrangement of lenses and the formation of images (Mudd and Anderson, *courtesy* Interscience Publishers Inc.)



(a) Light Microscope



(b) Electron Microscope

FIG. 2. Diagrams of light and electron microscopes. L_1 condenser lens. L_2 objective lens. L_3 eyepiece lens (Mudd and Anderson, *courtesy* R.C.A. Photophone Ltd.)

radiation, a condenser, an objective lens, and a projection lens or eye-piece (Fig. 2). It must, however, be emphasized that in the electron microscope the "lenses" are not solid structures of glass or quartz but fields of electric force. There are, moreover, other important differences. Since air itself scatters electrons a high vacuum must be maintained within the microscope: the electron beams are invisible and to aid in locating the specimen a fluorescent screen must be introduced, the permanent records being made on photograph plates; finally, and most important, the electron images are not due to the absorption and refraction of light rays, but to the scattering of electrons, and are therefore much more analogous to X-ray pictures than to photo-micrographs obtained from a light microscope; that is, the image formed depends upon the thickness and density of the object, not upon its powers of refraction.

Although electrons of very great speed, which may have wavelengths of a 1/100,000th that of visible light, can readily be obtained from a high voltage cathode-ray oscillograph, the aberrations of the "lens systems" of the electron microscope, particularly the spherical aberrations, are so great that in practice the resolving power is not 100,000 times that of the light microscope, but from 50 to 100 times: even this, however, permits of magnifications of up to 150,000 diameters as compared with 2000 diameters of the ordinary light microscope.

Although there is no satisfactory evidence that the electron beam of itself produces any morphological changes in the object under examination (but see Marton *et al.*, 1946), there are, nevertheless, certain drawbacks to the use of the electron microscope. The unavoidable presence of a high vacuum inevitably produces some degree of desiccation of the semi-fluid or potentially fluid inner protoplasm of the cell, and even actual distortion of the whole cell from shrinkage. But such changes are generally obvious. Again, biological particles of the size studied may occasion so little scattering of electrons that the resulting images are too lacking in contrast to be photographed satisfactorily. To overcome this difficulty attempts were made to increase the opacity of the particles by impregnating the object to be photographed with various radicles of greater electron-scattering power, e.g. osmic acid (Sharp *et al.*, 1944), calcium chloride (Sharp *et al.*, 1943), certain heavy metals (Mudd and Anderson, 1942). These measures, though

of some value, did not prove of great help, and, in the case of the smaller viruses, were definitely disappointing. A great advance was made by the development of Williams and Wyckoff (1945*a*, *b*, *c*, *d*) of a shadow-casting technique. This consists in depositing an extremely thin layer of metal (*circa* 7mμ) obliquely on the preparation to be examined; the distribution of this layer is determined by the contours of the specimen, relatively more metal falling on the more elevated parts, which in turn shield other areas —much as may happen with drifting snow. These shielded areas, deficient in deposited metal, form the "shadows" of the micrographs, and so give a brilliant three dimensional effect (Figs. 3 and 4). To quote from Williams and Wyckoff, "Just as an observer on a mountain-top or in a plane sees many details of a landscape at sun-rise or sun-set which would be invisible to him at noon, so metal shadow-casting brings out much detail that is not to be seen in an unshadowed (or in a vertically coated) preparation." Moreover, by varying the exposure, one can obtain pictures illustrating either the general shape of the particle or its more detailed surface-contour. But adequate differentiation of intracellular structures has still to be achieved.

Bacterial Cytology

The subject of bacterial cytology is one of quite peculiar difficulty: the literature is immense and often highly controversial; moreover, as Lewis (1941) has pointed out, there is probably no one fully competent to assess the worth of the many and often conflicting reports that have appeared. In any case, such an approach is quite outside the scope of this book, but those who are interested in the wider ramifications of the problem are referred to the review by Lewis (1941) and the monographs of Knaysi (1944) and Dubos (1945). All that will be attempted here will be to discuss the more recent work, particularly in relationship to investigation by electron microscopy.

And at once it must be confessed that so far the use of the electron microscope in bacteriology has posed new problems, rather than solved old ones. But this was to be anticipated. What it has also done is to place on a sound basis much of the older work on bacterial morphology, and furnish much new information on the ultra-microscopic viruses.

The fact that many bacteria are cylindrical rather than spherical

in shape indicates a certain rigidity of structure, but the presence of a rigid cell wall, though long inferred, was not established beyond doubt until confirmed by the very ingenious micro-dissections of Wámoscher published in 1930. Wámoscher worked with a large species of coliform bacillus—*B. mazun*—but similar firm, elastic outer sheathes have since been clearly demonstrated by electron microscopy in a wide variety of organisms—cocci, aerobic and anaerobic bacilli, fusiform bacteria and spirochætes (see Mudd and Anderson, 1944, for illustrations and references). In bacteria which occur in chains or clusters, or in organisms which have not separated completely, the cell-wall may remain intact as a delicate strand between adjacent cells (Johnson, 1944) (Fig. 5). Robinow (1944) by means of plasmolysis, has shown septa in giant forms of *B. megatherium*, which thus consist of several distinct but continuous cells (Fig. 6). Moreover, as Mudd and his collaborators have demonstrated, the cell-wall is sufficiently rigid not only to retain its contours when the inner protoplasm shrinks away or even escapes completely from a damaged cell, but also to fracture cleanly when it is broken by suprasonic oscillations (Mudd *et al.*, 1941).

Within, and anatomically distinct from the cell-wall, lies the fluid or semi-fluid protoplasm of the cell. Mainly from its staining reactions it was inferred that this was condensed into an outer layer—*the cytoplasmic membrane*—enclosing the more fluid inner protoplasm, but not sharply differentiated from it (Knaysi, 1941). According to Knaysi it is this cytoplasmic membrane which shows up so brilliantly under dark-ground illumination, which takes part in the ordinary staining reactions, and which is the site of the main metabolic activities of the cell. In later investigations, the same author (Knaysi, 1946) has shown that the cytoplasmic membrane is well differentiated, both physically and chemically, from the inner protoplasm, that it is of lipo-protein nature, and that it may form septa within the cell and so produce compartments which "are potential places of cell division." Moreover, and of importance from the strictly morphological point of view, he has been able (1947) to demonstrate the cytoplasmic membrane in some very beautiful electron micrographs of *B. mycoides* (Fig. 7).

Outside the cell-wall a third zone has been distinguished—the *slime-layer*. In the past it was believed that this outer layer was found in relatively few organisms, the so-called "capsulated species," but it now seems probable that most, if not all bacteria,

can secrete substances, largely of a carbohydrate nature, which condense to form "capsules' in widely-varying degree (Knaysi, 1938, 1941), and there is considerable indirect support for this (Miles and Pirie, 1939; Rosen, 1938). The capsule of the pneumococcus has been examined under the electron microscope and revealed as a gel of low density (Mudd, Heinmets and Anderson, 1943). Many bacteria, including cocci, are motile, and since the earliest days of bacteriology it has been universally accepted that the motile species possessed flagella and moved by means of them (Figs. 8 and 9). Although these structures have been beautifully demonstrated by electron micrography (Mudd and Anderson, 1942, 1944), even in organisms, such as *Tr. pallidum*, which were not before thought to be flagellated (Wile and Kearney, 1948; Mudd, Polevitzky and Anderson, 1943), it must be confessed that our ideas concerning them have undergone very considerable modification in recent years. And this change has been almost entirely due to the work of one man—Adrianus Pijper.

Using a highly-developed dark-field technique with the sun as a source of illumination, Pijper (1938) originally showed that in *S. typhi* and certain related species the flagella were not peritrichous, as had long been believed and repeatedly illustrated in text-books, but were arranged in two rather coarse spiral structures; during active locomotion these became loosely coiled into an organ analogous and very similar to a tadpole's tail. On the death of the organism the two main "flagella" would separate out into numerous fine threads; but Pijper, even at this time, clearly regarded the classical peritrichous flagella seen in stained specimens as artefacts —a view that was to receive some confirmation by the work of Pietschmann (1939) and the electron micrographic studies of Hofer (1944).

Pijper's subsequent investigations (1941*a* and *b*, 1945, 1946), however, have led him to still more heterodox opinions; but opinions, it should be added, which are founded on careful observation and logical argument, and backed by excellent illustrations. His view is that the tadpole-like tails which he has repeatedly photographed are really the fluttering edges of the slime-layer, trailing behind the bacterium in consequence of its own movements. Thus the flagella are the result and not the means of motility. By suspending his organisms in a solution of methyl-cellulose Pijper (1946) was able to slow down their movements sufficiently

to examine them directly, and by these observations and by numerous cinematograph pictures he has convinced himself that the bacilli move by "wave-like spiral contractions"—in fact much as a water-snake or eel swims. It is these gyratory movements which twist the slime-layer into "tails" or even into fine wavy threads. Moreover by special methods of culture Pijper has been able to obtain motile bacteria which are non-flagellated.

As against these claims it may be mentioned that Johnson, Zworykin and Warren (1948) have published an electron micrograph (Fig. 11) in which flagella are apparently still attached to the cell protoplasm after the cell-wall has become in part detached; and more recently Ørskov (1947) has described how in a special indian-ink agar medium bacteria can be seen to clear away the carbon particles immediately surrounding them—presumably by the action of their flagella.

Yet these are not very serious contradictions, and, however difficult it is to believe that appearances such as those seen in the illustrations (see also Fig. 10) are mere artefacts, it must be conceded that the arguments and illustrations Pijper adduces are most convincing. These are too lengthy and complex to detail here, but all who are interested should study the original papers and in particular his latest (1946) communication.

If this most stimulating theory be correct it will, of course, solve many long-standing problems concerning bacterial flagella (*Lancet*, 1946), but it also poses a new and more difficult question—what is the essential difference in structure between, say, a typhoid and a dysentery bacillus, which permits one to twist and spin and forces the other to remain rigid and still?

So far as the intra-cellular structure of bacteria is concerned there has been a great revival of interest in recent years, particularly regarding the presence of a nucleus. This question, one of the most debated in morphological bacteriology, has been reviewed in detail by Lewis (1941), who lists no less than eight different hypotheses, each supported by a large number of investigators over a long period of years. And in an elucidation of the problem the electron microscope has not as yet proved of much assistance. Nor is this very surprising; in this case the trouble is not so much the small size of the objects under examination, but the difficulty of deciding which nucleated micro-organisms are in actual fact bacteria, and (as Knaysi, 1938, first pointed out) what are the criteria by

which an atypical nucleus may be identified. In 1942, this author suggested that intra-cellular granules which possessed most or all of the following properties should be considered to be of nuclear nature. They ought to give a positive reaction when tested for the presence of nucleo-protein; they must be adequately differentiated from nutritive particles of fat, lipoprotein, glycogen, and, above all, volutin; they must be present in young cells and old cells, and in well-nourished and in starving cells; and they should be present during the period of active growth, and should divide before the cell divides. The stringent application of these conditions at once rules out conclusions based on much of the earlier work on the subject, but there can be little doubt that in some bacteria structures satisfying all these criteria have been unequivocally demonstrated.

Thus Knaysi (1942) identified a nucleus in the cells of a staphylococcus—*Staph. flavo-cyaneus*—by all the methods detailed above, and these investigations were later confirmed and extended by Knaysi and Mudd (1943), who repeated Knaysi's original experiments, and also, by rupturing the cells of *Staph. flavo-cyaneus* by suprasonic vibrations and subsequent high-speed centrifugation, obtained the nuclear granules in relative purity. They found these contained deoxyribo-nucleic acid, purine nitrogen and phosphorus. Moreover, examination under the electron microscope revealed that in actively growing cells the granules became reduced in size, probably owing to part of the nuclear material going into solution. Similar granules were demonstrated in a strain of *N. meningitidis*, but not in *N. gonorrhœæ*, *Staph. aureus* or *Strept. pyogenes*. Certain opaque areas seen in cells of *P. pestis* and *Br. abortus* were believed to be reserve food material rather than nuclei. It is noteworthy that the strains of *Strept. pyogenes* studied were those already examined by Sevag, Smolens and Lackman (1940) and known to contain a considerable amount of thymonucleic acid. From these findings they concluded that the nuclear apparatus of bacteria varied markedly with the species, in some being highly differentiated, in others dispersed throughout the cell.

Later work by Knaysi and Baker (1947) has, however, considerably clarified this problem. He found that if spores of *B. mycoides* were seeded into a nitrogen-free medium, many of them would germinate. The resulting bacillary cells grew at the expense of

nitrogenous material—probably volutin—diffused in their cytoplasm: as this was used up, internal differentiation became much more striking, so that distinct bodies, endowed with those nuclear criteria which Knaysi (1942) had already postulated, became clearly visible, even under electron-microscopy. It is evident that further development of this technique and its application to other species of bacteria should be of great interest.

Robinow (1942, 1944, 1945) has approached the problem with a different technique, but with results which are as suggestive as those of Knaysi. Developing the work of Piekarski (1937, 1938, 1939, 1940), Neumann (1941) and others, who had been able to demonstrate nucleus-like bodies in various species of bacteria following treatment with dilute hydrochloric acid, he evolved a modification of the Giemsa stain which clearly demonstrated dumb-bell-shaped chromatinic bodies lying transversely in the cells of a large number of bacterial species. In old cells these bodies were small and difficult to demonstrate, but in young actively growing cultures they were very obvious (Fig. 12) dividing longitudinally (i.e. transversely in the bacillary cell) before the whole organism divided, so that "multinucleated" forms were frequently found (Fig. 13). The close co-relation between the growth and multiplication of these bodies and that of the whole cell, their resistance to acid hydrolysis and their positive reaction to micro-chemical tests for the presence of nucleo-protein make it highly probable that they are of a nuclear nature. To quote from Robinow (1945) "these observations suggest very strongly that the chromatinic rodlets are integral constituents of the bacterial cell and that they may reasonably be regarded as the bacterial cells equivalent of *chromosomes*."

Much of this work on the finer structures of bacteria is too intricate to describe in detail, and the original papers should be consulted by those interested. Its importance lies in the unequivocal demonstration of comparable nucleoid bodies in a large number of bacterial species by many independent investigators, using different experimental methods. To sum up: it seems certain that in many bacteria a true nucleus exists; in others the nuclear material cannot be demonstrated by present techniques and may be diffused throughout the cell. But the highly specialized cytological investigations necessary have not yet been sufficiently widely applied to permit of any generalization on the subject.

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