BRITISH MEDICAL JOURNAL

LONDON SATURDAY DECEMBER 3 1949

ELECTRON MICROSCOPY OF BACTERIA AND VIRUSES*

BY

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[WITH PHOTOGRAVURE PLATE]

It is not proposed here to discuss the electron microscope itself, but the reader should be aware that specimens must be dried for examination in this instrument. Also, since there are no electron-transparent materials, the supporting "slide" must be made extremely thin, and for most purposes a film of nitrocellulose about 20 m.μ in thickness is used.

Electron microscopy, like other forms of microscopy, has its artifacts. Circular objects may appear oval because of astigmatism in the projector lens or because of defective cleaning or alignment of the microscope. Specimens may be damaged by the heat of the electron beam but not show the usual signs of charring which follow burning in air. In the case of lipid granules, for example, a clear-cut hole may be created in the object by volatilization (Ruska, 1942). Usually the damage is not so obvious as this, and may take the form of a reduction in size by surface evaporation (Marton *et al.*, 1946), or even of an increase due to condensation on the object of carbon from the oil of the diffusion pump or of materials volatilized in hotter parts of the field (Watson, 1947; Cosslett, 1947). These changes in size seldom if ever occur uniformly over the surface, the result usually being that the object acquires a somewhat "moth-eaten" appearance.

The temperature attained by the object depends also on the rate of dissipation of heat by radiation and conduction. This is usually adequate in the case of the thinnest objects —e.g., plant viruses, flagella, and fibrils of all kinds, which can be examined unsupported or on the conventional film of nitrocellulose. Von Ardenne (1948) has successfully created convection around thick objects by means of a stream of hydrogen molecules not in itself sufficient to affect the vacuum in the microscope seriously. With the additional help of a screen to shield parts of the object field not actually under observation he has photographed crystals of sulphur without sublimation occurring.

Metal-shadowing

Heating artifacts are most serious after metal-shadowing. In this procedure (Williams and Wyckoff, 1946) a heavy metal is volatilized in a vacuum chamber and the metal atoms, travelling in straight paths, fall obliquely on the specimen. The metal builds up on the exposed side of the object, a shadow resulting in the background and a relief effect being created. In this way height is used to obtain contrast in biological objects which are notoriously "empty" after drying. Now a vacuum-deposited metal layer, however carefully it is produced and however highly reflecting it appears to the naked eye, is not coherent at the high magnifications of the electron microscope. It consists of discrete crystallites, and the best efforts of the shadowing expert are directed towards achieving the smallest size for these, since clearly this affects the resolution of the final picture. Having once obtained a metal pattern that is fine enough, there is also the problem of preserving it, because under the influence of the electron beam neighbouring crystallites tend to fuse. This change can often be observed in progress, and only speed in exploring the field and in focusing will prevent it. Where fusion of metal occurs across an elongated object the impression is created of a chain of objects (Mandle, 1947).

Artifacts Due to Drying

Of greater practical significance in the biological field than all other artifacts are probably those due to drying. In spite of attempts to examine sealed wet specimens, there is at the present time no practical alternative to complete desiccation. Opinions differ on how the drying should be carried out—whether slowly or quickly, in air or *in vacuo*. Wyckoff (1946) recommends drying from the frozen state, and the underlying principle seems to be sound—namely, that sublimation of ice from a truly frozen object will leave all the non-volatile constituents in their natural spatial relationships. However, the effect on objects of low hydration—e.g., plant viruses—is not noticeable; and on others in which the degree of hydration is doubtful—e.g., influenza virus—it is surprisingly small. There seems to be no recorded case of its successful application to highly hydrated objects, for which there is plenty of evidence of shrinkage in the electron microscope, and the following experience (I. M. Dawson and A. S. McFarlane—unpublished) probably supplies the reason.

A drop of a suspension of vaccinia virus weighing less than 50 mg. was frozen on a specimen disk and placed in a jacket at $-76°$ C. The water vapour was pumped away over a period of 24 hours, calculation and experiment both showing (Tschudin, 1946) that this interval is necessary for complete drying because of the low vapour pressure of ice at $-76°$ C. To the naked eye the drop did not contract, but in the electron microscope nothing could be seen. The virus particles after drying are supported by delicate fibrils of residual protein and are so effectively insulated that they volatilize in the electron beam or are dispersed by it. This may also be the fate which is in

*Almroth Wright Lecture delivered at the Wright-Fleming Institute of Microbiology, St Mary's Hospital, on June 7, 1949.

store for any truly frozen-dried cell in the electron microscope, and it seems that a practical limitation may thus be placed on the technique. Fortunately, in most cells shrinkage due to drying is greatly reduced by preliminary use of a protein fixative, osmic acid being particularly effective.

Finally, it seems to me that the stricture sometimes passed on electron microscopy—namely, that it deals with distorted desiccated residues which give little or only misleading information—is altogether too severe. The effects of drying are not entirely haphazard, and we are already able to make allowances for some of them. Micrographs of many highly hydrated objects contain much information which cannot be obtained otherwise, but a considerable amount of deductive reasoning is necessary to extract it.

Plant Viruses

It is fortunate that the electron microscope came to perfection at a time when great progress was being made in the study of plant viruses, since in many ways these nucleoprotein macro-molecules are ideal objects for study. Fig. 1 (Plate) shows a crystal of tobacco necrosis virus prepared by Smith and Markham and photographed by Wyckoff (Markham *et al.*, 1948). It is, however, a kind of fake, since the crystal itself is opaque to electrons and was not in fact photographed. Instead it was covered by a layer of collodion, which follows the surface topography of the crystal faithfully right down to molecular dimensions and retains the impression after it is stripped off. However, the film is almost devoid of contrast, and so this is created by metal-shadowing. Virus molecules approximately 24 m.μ in diameter are seen in a perfectly regular arrangement in the crystal lattice. In general, studies of this kind have provided striking confirmation of information obtained indirectly by the crystallographer by means of *x*-ray diffraction.

However, the electron microscope often provides a unique kind of information. In Fig. 2 is shown the expressed juice of an infected tobacco plant (*a*) fresh and (*b*) after standing at 4° C. for 20 days (Sigurgeirsson and Stanley, 1947). On ageing, the tobacco mosaic virus rods have joined up mainly in pairs, and it is a matter of some general interest that the joint appears to be as strong as the component rods. Various other physical methods are able to tell us that the mean rod-lengths are 280 and 560 m.μ respectively in the two preparations, but only the electron microscope can provide the information that variations about the mean are small enough to be consistent with the idea of rod-shaped molecules. Hitherto the possibility existed that the virus consisted of fibres of variable length but of uniform cross-section. Incidentally, also, particles in the field smaller than 280 m.μ are non-infective and have some as yet undefined relation to the virus.

Fig. 3 illustrates a further technical point connected with plant viruses. It shows the tomato aucuba mosaic virus photographed by Cosslett (1948) on a supporting film of beryllium. This metal has a low atomic number and low density and therefore scatters electrons less, for example, than does nitrocellulose. It is deposited *in vacuo* vertically on to the specimen on a soluble base, and on dissolving away the latter the specimen on beryllium is handled much as one handles a collodion film. The result is a picture of the virus rods showing excellent contrast.

Bacteriophage

In the field of bacteriophage the electron microscope reveals that these minute bodies have in some cases heads and tails (Fig. 4), and the heads may be differentiated into a dense centre and a lighter periphery. It is difficult to see how this important information could have been obtained in any other way. The heads vary from 30 to 90 m.μ in diameter, and the tails vary both in length (up to 250 m.μ) and in width (up to 20 m.μ) (Ruska, 1943; Anderson, 1943). Ruska (1942) and Kottmann (1942) find phage particles free in the culture medium, as well as closely packed on the surface of the host cell, but they find no evidence of multiplication taking place inside the bacterium. They visualize the tailed phages as approaching the host cell head first, the tail swinging round and penetrating the cell wall to form a link between the head of the phage and the bacterial cytoplasm. This seems not unreasonable in view of the manner in which flagella are seen to pass through the cell wall and into the cytoplasm (Van Iterson, 1947). Multiplication is believed to proceed on the surface of the host cell until the metabolism of the latter is so weakened that it undergoes lysis.

Wyckoff (1948a, 1948b, 1948c, 1948d) equally strongly supports the opposite point of view. He studies mainly phages on a solid medium, stripping a superficial layer from the medium by means of collodion, and finding phage bodies on this as well as the remains of lysed bacteria (Fig. 5). He believes not only that the phage multiplies inside the host cell but that most of the host cell materials are used up in the process. We may reasonably expect that in the near future further electron microscope studies will resolve this important controversy.

Animal Viruses

In the field of animal viruses, round (Jungeblut and Bourdillon, 1948) and filamentous forms (Tiselius and Gard, 1942) of murine poliomyelitis virus are described, a difficulty in the way of accepting the latter being that similar filaments are found in the stools of normal mice (Melnick, 1944). Eastern and Western equine encephalomyelitis are both approximately spherical and only 40–50 m.μ in diameter (Sharp *et al.*, 1943). Fig. 6 shows evidence of differentiation in the particles not unlike that seen in some phage heads. This seems to disappear when contrast is artificially increased by suspending the virus in a weak solution of calcium chloride.

The fact that no micrographs of the smaller viruses, or only unsatisfactory ones, are available is not altogether due to inability to prepare specimens pure enough; it may also be connected with the practical resolution of the microscope. The theoretical limit of visibility using 50 kV electrons according to Abbé's law is in the region of 0.005 m.μ, but the practical limit because of numerous residual aberrations is in the region of 1–2 m.μ for objects of good contrast on a clear background (Hillier, 1946). Although some biological objects—e.g., plant viruses and bacterial flagella—satisfy these conditions and photograph with approximately this resolution, the hydrated ones in general do not. In our experience an object in this category of 200–300 m.μ diameter photographs in ultra-violet light with about the same resolution as does one of 20–30 m.μ in the electron microscope. This suggests a much smaller factor—namely, approximately 10—for the superiority of the electron microscope over the best alternative instrument for studying many biological objects.

Recent electron micrographic studies raise acutely the question of pleomorphism in animal viruses. The round and filamentous forms of poliomyelitis have already been mentioned. The virus of Newcastle disease grown in the developing egg has a sperm-like form (Cunha *et al.*, 1947), which is believed by some (Elford *et al.*, 1948) to arise by degeneration of an originally spherical body. Filamentous

A. S. McFARLANE: ELECTRON MICROSCOPY OF BACTERIA AND VIRUSES

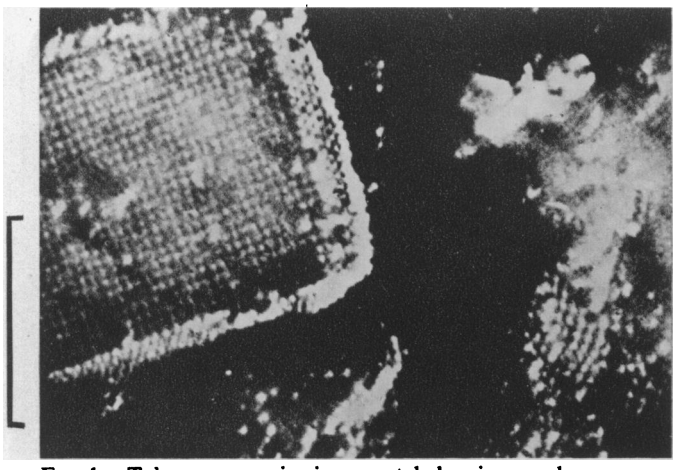

FIG. 1.—Tobacco necrosis virus crystal showing regular arrangement of virus particles of 24 m.μ diameter in a cubic close-packed lattice.

FIG. 2.—Expressed juice of mosaic-diseased tobacco plant (*a*) before and (*b*) after standing for 20 days at 4° C., showing linear aggregation of virus rods.

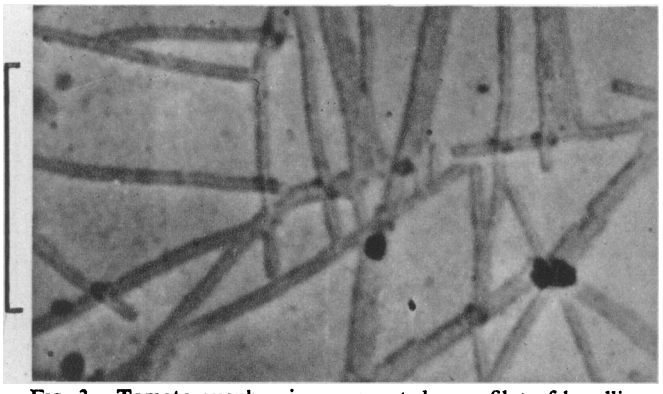

FIG. 3.—Tomato aucuba virus supported on a film of beryllium.

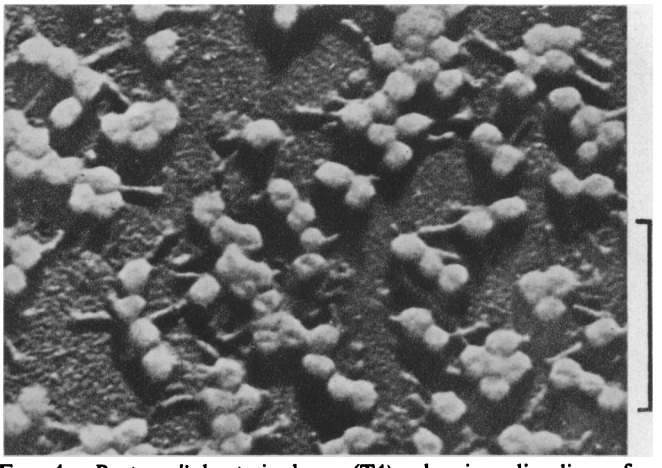

FIG. 4.—*Bact. coli* bacteriophage (T4), showing dimpling of heads and small knobs on the ends of some tails.

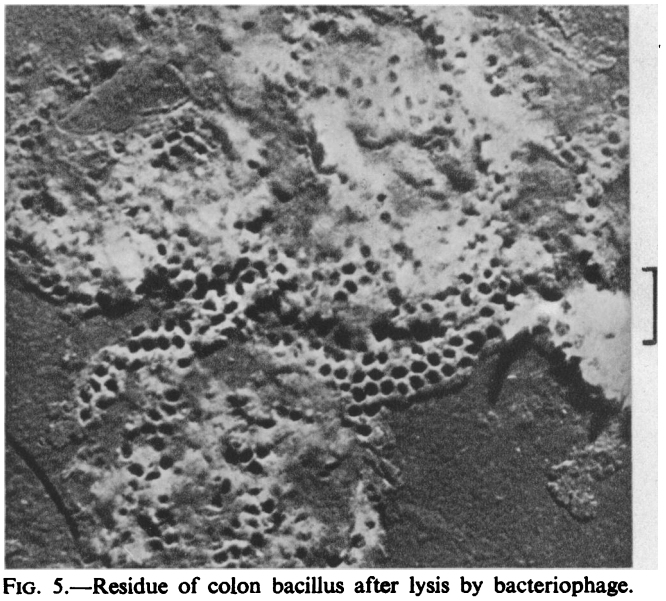

FIG. 5.—Residue of colon bacillus after lysis by bacteriophage.

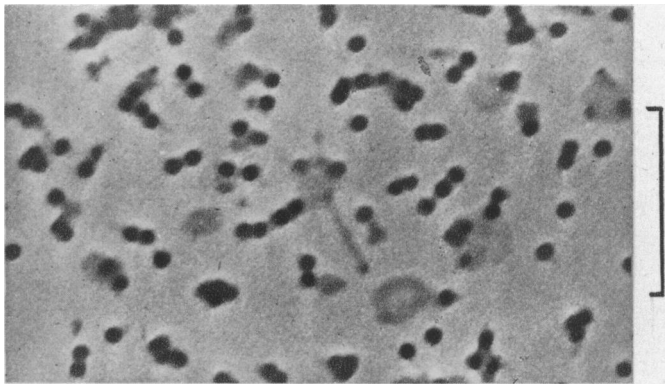

FIG. 6.—Eastern equine encephalomyelitis virus. Spherical dense bodies enveloped in material of irregular shape and much lower contrast.

Each marginal line (⌐¬) represents 1μ

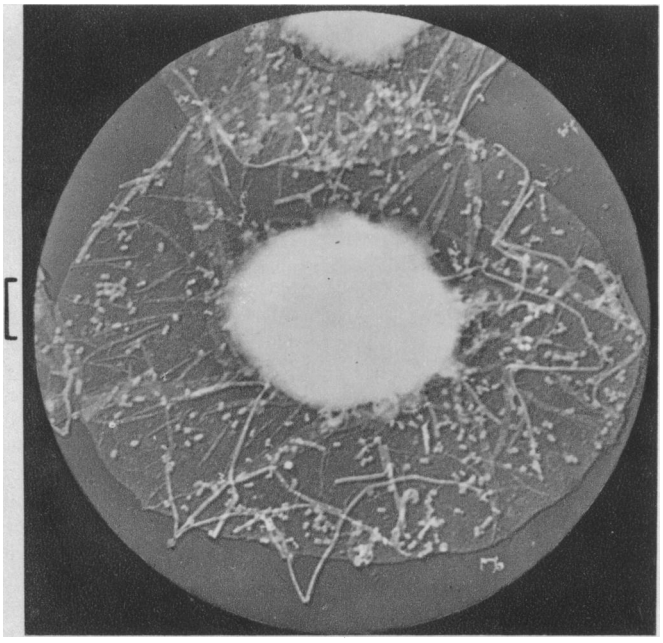

FIG. 7.—Influenza virus adsorbed on a laked avian erythrocyte. Central mass is erythrocyte nucleus. Filaments and spherical forms of virus are seen on outer erythrocyte membrane.

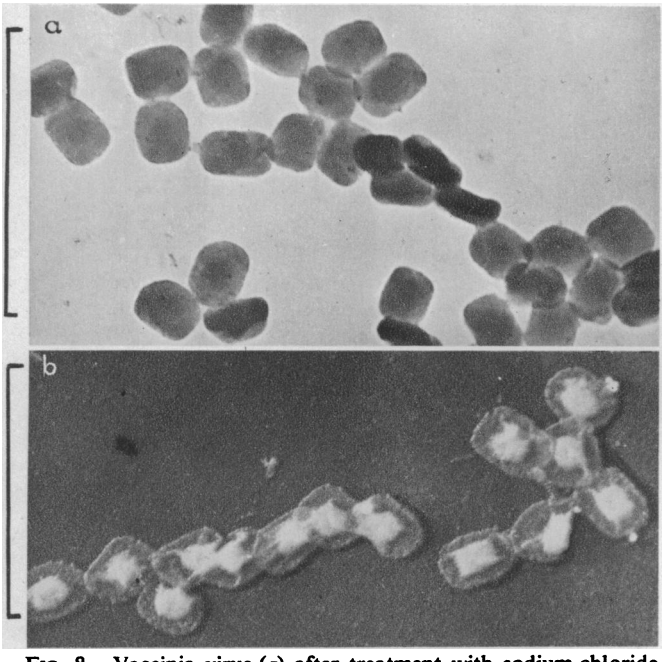

FIG. 8.—Vaccinia virus (*a*) after treatment with sodium chloride solution; (*b*) after digestion with pepsin and gold shadowing, showing "nucleus" and outer membrane.

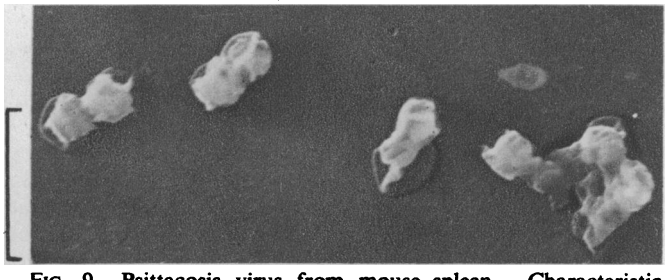

FIG. 9.—Psittacosis virus from mouse spleen. Characteristic puckered surface, periphery of outer membrane lying flat on supporting film.

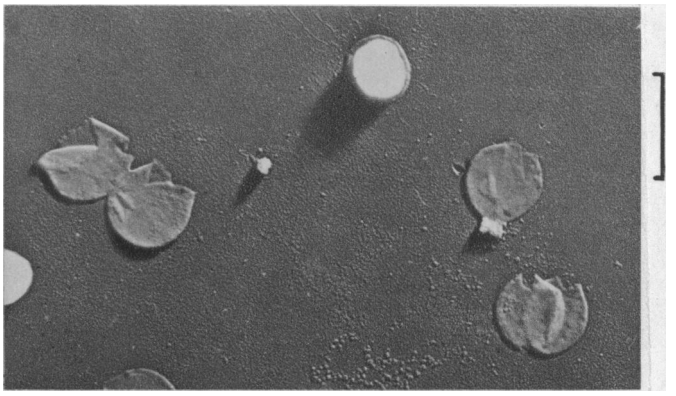

FIG. 10.—*Staph. aureus* after mechanical treatment to disrupt shells. One intact coccus (top centre); remainder are empty shells.

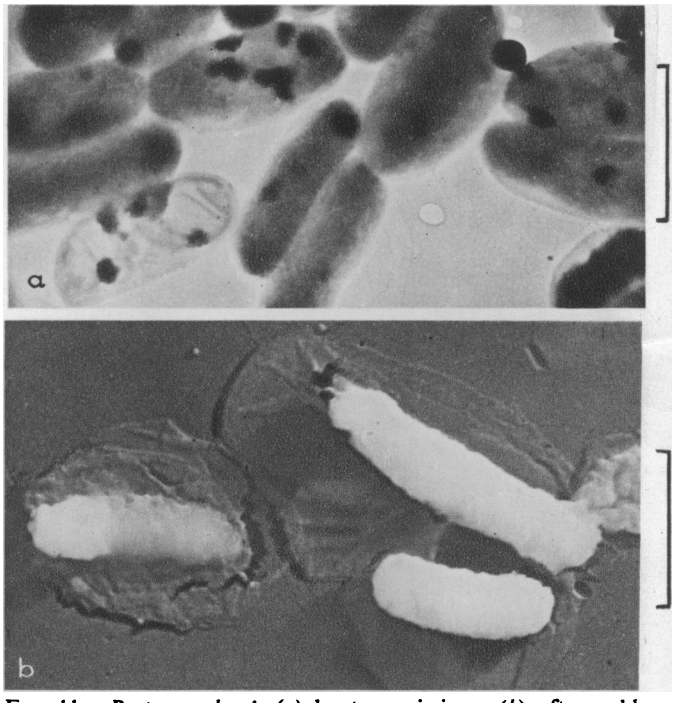

FIG. 11.—*Proteus vulgaris* (*a*) by transmission; (*b*) after gold shadowing, showing wide variety of forms—some relatively empty with small inclusions and others with marked contraction of bacterial cytoplasm.

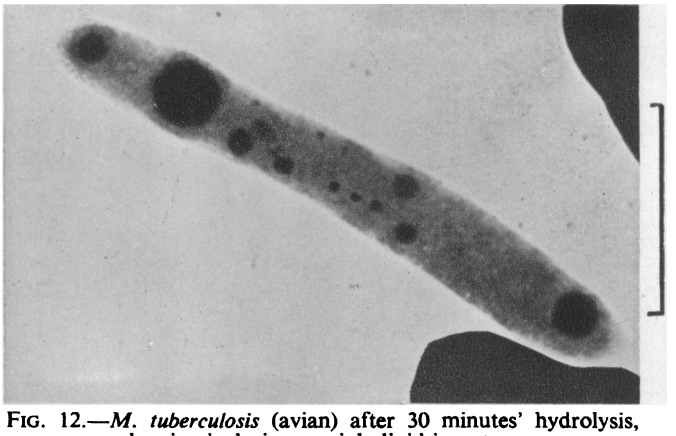

FIG. 12.—*M. tuberculosis* (avian) after 30 minutes' hydrolysis, showing inclusions mainly lipid in nature.

Each marginal line (⌐¬) represents 0·5μ

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and spherical forms are also observed of fowl plague (Dawson and Elford, 1949) and influenza viruses (Mosley and Wyckoff, 1946; Heinmets, 1948; Chu *et al.*, 1949) grown in the egg. Heinmets regards the influenza filaments as degenerate forms of the spherical virus, while Chu *et al.* (1949), who find them (Fig. 7) in remarkably high proportion in some recently isolated strains of the virus, interpret their evidence as suggesting that they represent a stage in virus multiplication. Clearly only infectivity tests can decide here between non-specific fibrils and pleomorphic forms of the virus.

Viruses of the Pox Group

In the pox group of viruses, which have a characteristic brick shape (Fig. 8, a), are included the causal agents of vaccinia moluscum contagiosum, ectromelia, rabbit myxoma, canary pox, and fowl pox (Ruska and Kausche, 1943; Boswell, 1947; Nagler and Rake, 1948). Size variations in colonies of the same virus are of the order ±25%. It cannot be said with certainty that the brick shape is characteristic of the wet virus as it is of the dry, but most of the evidence is in favour of this. Contraction on drying appears to take place mainly along the vertical axis of the brick. There is also a faint suggestion of internal differentiation (Green *et al.*, 1942), which is confirmed in vaccinia by digesting the virus with pepsin (Dawson and McFarlane, 1948). The biochemical findings in this experiment are unequivocal, more than three-quarters of the virus substance and none of the phosphorus or nucleic acid being removed and the action of the enzyme being sensibly complete in half an hour.

A limiting membrane is clearly revealed (Fig. 8, b), and it seems justifiable to speak of this virus as having a nucleus and a cytoplasm without implying that the mechanism of reproduction is necessarily by mitosis. Indeed, nothing in the electron microscopy of animal or plant viruses has, in my view, yet provided a useful clue to their manner of self-duplication, although there are some claims to the contrary. In shadowed pictures of vaccinia virus "purified" by salt flocculation the surface of the virus appears granular, and in places the granule arrangement has a certain regularity but of a much lower order than that seen in the tobacco necrosis crystal. Nevertheless, it raises the possibility that the cytoplasm of this virus is a gel-like assemblage of large protein molecules, not unlike the cytoplasm of the erythrocyte (Perutz, 1948), and, like it, prone to a regular arrangement of all or part of its protein molecules (Teitel-Barnard, 1932). Considerable disorientation may be expected to occur on drying.

Viruses of the pox group never show the dimpling or indentation of the surface which characterizes the feline pneumonitis–psittacosis–lymphogranuloma group. Electron micrographs (Rake *et al.*, 1946; Hamre *et al.*, 1947; Kurotchkin *et al.*, 1947) (Fig. 9) reveal the members of this group to be approximately spherical in outline, about 300 m.μ in diameter, and with a rather larger variation in size than is the case with the pox group. Occasionally very large forms (about 800 m.μ in diameter) are seen, such as do not occur in the pox group. However, probably of greater significance is the complete resistance to the action of proteolytic enzymes (C. F. Barwell, I. M. Dawson, and A. S. McFarlane—unpublished), and the marked electron-microscope signs of shrinkage on drying. The impression is created of hydrated bodies with rather thick limiting membranes or capsules, and there is evidence that these properties are shared by the rickettsiae (Plotz *et al.*, 1943; Weiss, 1943; Eyer and Ruska, 1944) and by some bacteria (I. M. Dawson and A. S. McFarlane—unpublished).

Opacity

Early pictures of many bacteria in the electron microscope were somewhat spectacular but on the whole remarkably uninformative. It is clear that nearly all young organisms are too opaque to give more than shadowgrams (Piekarski and Ruska, 1939). However, some information is forthcoming. To quote a few examples, by examining *Bact. chromoprodigiosum* in a surface culture with the minimum of disturbance Ruska (1941) demonstrated that the pigment granules (prodigiosin) are released from the surface of the intact organism and are not liberated by lysis. It is possible to release the contents of *Staph. aureus* by mechanical means and to leave well-formed cell walls or shells behind (Cooper *et al.*, 1949) (Fig. 10). Whereas the bodies of capsulated pneumococci are extremely opaque the capsules differ from the cell walls of staphylococci in having little or no contrast, and the conclusion must be that these are highly hydrated and probably semi-fluid polysaccharide gels (Mudd *et al.*, 1943). Flagella, varying widely in length and thickness, photograph extremely well, and it is clear as a general rule that they pass through the cell wall and are not to be regarded as appendages of it (Van Iterson, 1947).

The opacity of large objects is reduced by the use of high-voltage electrons. For instance, granules appear in sarcinae at 110 kV which are not visible at lower voltages (Van Iterson, 1947). In general, also, the heating effect is less with fast electrons, so that there appears to be considerable scope for development of high-voltage instruments in the biological field, especially for examining thick specimens or shadowed replicas.

In general, the opacity of cocci of all kinds persists at all ages. On the other hand, many bacteria, and especially the non-spore-bearers, become partly transparent in older cultures (Fig. 11, a), and these are classed by Piekarski and Ruska (1939) as secondary forms. Shadowed pictures (Fig. 11, b) reveal this change to be in many cases merely a question of shrinkage of the cytoplasm, since the dark central areas cast proportionately longer shadows the smaller they are. The significance of this detachment from the cell wall in the life cycle of the organism is not known, nor whether it is present before drying. Bacteria of reduced contrast and showing no signs of shrinkage can often be seen in the same field. Why the cytoplasm shrinks away from the cell wall in some organisms while retaining most of its solids, and why in others there occurs a uniform loss of solids with no shrinkage or distortion of the cytoplasm, is not understood.

Development of Secondary Forms

How the secondary forms further develop is also not clear. Some of course die, and we presume this to be accompanied by loss of contents, leaving empty shells. In spore-bearers progressive increase in shrinkage may be preliminary to spore formation. The remainder, which proceed to cell division, must presumably rehydrate in some manner. After cell division sharply defined inclusions can sometimes be seen which are both lighter and darker than the surrounding cytoplasm (Ruska, 1942). Some bacteria are exceptional in showing similar inclusions early in the life of the organism. *M. tuberculosis* has been an object of special study (Lembke and Ruska, 1940; Mudd *et al.*, 1942; Brieger *et al.*, 1947) in this connexion, and treatment with lipid solvents reveals that the dark clean-cut inclusions (Fig. 12) are in this case for the most part lipid in nature (Lembke and Ruska, 1940). No signs are seen of the classical waxy envelope which has

been postulated as the basis of the acid-fast character of these organisms. There is little doubt that granules consisting predominantly of proteins, lipids, or polysaccharides occur irregularly in many organisms and in different metabolic states of the same organism.

These considerations naturally provoke speculation about the status of the nuclear bodies stained by Stille (1937), Piekarski (1937, 1939) and Robinow (1939, 1942) in numerous bacteria and which have come to occupy a somewhat controversial position in bacteriology in recent years. It is quite clear that electron microscopy does not settle the issue, since many organisms which show such bodies when stained by the Feulgen or Giemsa technique are too opaque for study with electrons. On the other hand, it is possible that some of the stained organisms are secondary forms, and what is taken to be a nucleus is in fact the shrunken body of the organism, especially since the cytoplasm of most bacteria is diffusely basophilic. In the few cases (Piekarski and Ruska, 1939; Robinow and Cosslett, 1948) where the same organism has been stained and examined in the electron micrograph a somewhat surprising result has been obtained—namely, where the nuclear body occurs in the stained specimen a lighter vacuole-like area appears in the electron microscope. This suggests that the true nuclear inclusion is more hydrated than other inclusions, and more so even than the surrounding cytoplasm. While such a state of affairs is contrary to general cytological experience, it is perhaps not surprising to find it in organisms as dense as are most bacteria.

Observations have as yet been too few to allow generalizations to be made regarding the morphology of bacteria. It is clear that the greatest single difficulty in the way of progress lies in the variability of organisms of the same culture.

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CLINICAL TESTS OF A NEW COUMARIN SUBSTANCE

A REPORT TO THE MEDICAL RESEARCH COUNCIL

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This paper records the results of clinical tests of a new coumarin product, bis-3,3′-(4-oxycoumarinyl) ethyl acetate* (referred to here as B.O.E.A.), which seems to be a step nearer the ideal coumarin substance than any so far available for clinical use. Rosicky (1944) assumed that the delay in action of dicoumarol was due to difficulty in splitting the molecule, a biological fact later confirmed experimentally by Pulver and von Kaulla (1948). Rosicky therefore weakened the methylene linkage between the two coumarin groupings by the addition of a carboxyl group. Chemically the substance so produced was the ethyl ester of di-4-oxycoumarinyl acetic acid. Weight for weight this substance (B.O.E.A.) is about four times less active than dicoumarol — 3,3′-methylene-bis-(4-hydroxycoumarin) — 100 mg. of the latter corresponding approximately in anticoagulant action with 400 mg. of the former (Reinis and Kubik, 1948). B.O.E.A. is therefore prepared for clinical use in tablets of 0.3 g. for oral administration.

The comparative rates of action and excretion are set out in Fig. 1, which shows the average effect of a single dose of B.O.E.A. and of dicoumarol on the prothrombin levels in six subjects. In this group, after a single dose of B.O.E.A. the minimal prothrombin level was reached between eight and 24 hours and equally rapidly returned towards normal. With dicoumarol the minimal level was not reached for 32 hours and was maintained for a

*We wish to acknowledge the courtesy of SPOFA (United Pharmaceutical Works), Prague, and of Pharmaceutical Laboratories, Geigy Ltd., Manchester, who supplied this substance for trial under the respective names of "pelentan" and "tromexan."