THE INTERNAL STRUCTURE OF CERTAIN BACTERIA AS REVEALED BY THE ELECTRON MICROSCOPE—A CONTRIBUTION TO THE STUDY OF THE BACTERIAL NUCLEUS

GEORGES KNAYSI AND STUART MUDD

The Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca; the Department of Bacteriology, The School of Medicine, University of Pennsylvania, Philadelphia; and the Research Laboratories, RCA Manufacturing Company, Camden, N. J.

Received for publication September 8, 1942

The problem of the existence of a nucleus in the bacterial cell has aroused considerable interest among biologists and has been the subject of numerous investigations. A study of the literature (Knaysi, 1938, 1942a, Delaporte, 1939–1940, Lewis, 1941) shows that the methods used are various and in some cases ingenious, and that advantage has been taken of developments in other fields of endeavor (Kruis, 1913, Feulgen and Rossenbeck, 1924, Holweck, 1929, Lacassagne, 1929, Piekarski, 1939, Knaysi, 1941). Unfortunately, the net result of this collective effort has been a series of hypotheses ably reclassified by Lewis in his recent review (1941). Confusion has been added by the fact that certain investigators long associated with certain hypotheses have abandoned part or all of their former views. The causes of this confusion have been discussed by Knaysi (1938) who pointed out that they are to be sought, not only in the small size of the bacterial cell, as is generally believed, but principally in the absence of a single criterion by which an atypical nucleus can be recognized. Consequently, an analysis of the problem led him (1942a) to adopt the following criteria: (a) nucleoprotein nature of a structure, and, (b) behavior of that structure during growth and multiplication, in age and in starvation. Granules which gave a number of tests characteristic of nucleoprotein, which were present in young and old cells and in well-nourished and starved cells, and which did not correspond to any reserve material known to occur in bacteria, were found in *Staphylococcus flavo-cyaneus*. Moreover those granules were evident during growth of the cell and divided before cell division. Knaysi concluded that those granules represented the cell nucleus.

MICROORGANISMS

The above findings made it appear desirable to reinvestigate with the electron microscope the cell structure of *Staphylococcus flavo-cyaneus*. Moreover, it was decided to reinvestigate by the same means other microorganisms, because previous observations (Mudd and Anderson, 1942) had indicated that their cells either showed internal structures, as in the case of strains of *Neisseria meningitidis*, *Brucella abortus*, and *Pasteurella pestis*, or that they appeared uniformly opaque when observed at 60 kv., as in the case of *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Streptococcus pyogenes*, strains 1048M and C203M, (cf. Mudd and Lackman, 1941).

INSTRUMENTS AND METHODS

The electron microscope generally used in the present investigation was the RCA 60 kv. commercial model, but occasionally we were fortunate enough to have access to the high voltage, experimental model (Zworykin, Hillier, and Vance, 1941) which gives greater penetration. This characteristic is especially valuable in the study of cells which, heretofore, have proved to be opaque to electrons at the accelerating potential of 60 kv.

The methods used in preparing the material for observation were either one of the following: (a) the usual procedure (Marton, 1941) of suspending the organisms, taken from a solid medium, in sterile distilled water in a test tube and of transferring a small droplet to the surface of the collodion film over which it is spread and allowed to dry. We found it preferable to transfer the droplet with a small loop rather than to use a capillary pipette; (b) When the number of organisms on the solid medium was too small for a satisfactory suspension, we suspended the cells directly on the collodion film, by placing a small loopful of sterile, distilled water on the surface of the film and transferring the cells into the water droplet with a fine needle; (c) The cells from a culture on solid medium are smeared on a glass slide as if for observation with the light microscope. A drop of sterile, distilled water is deposited on the smear and the cells are resuspended with a small loop. The rest of the procedure is as before. This technique is to be recommended when the cells are to be given a certain treatment before observation and when a time-temperature factor is to be considered.

RESULTS AND CONCLUSIONS

Staphylococcus flavo-cyaneus. Figure 1 to 4 were taken with the 60 kv. microscope. Figure 1 shows that the untreated cells of this organism are rather opaque to electrons at this voltage. In general, the existence of an intracellular structure is suggested, but the picture is not sufficiently clear for an exact study of the form, position, and number of the granules. Staining for a minute or two with Nicolle's carbol thionine with subsequent decolorization with 0.1–0.15 per cent solution of acetic acid in water, a technique which was found by Knaysi (1942b) to be excellent for the demonstration of the granules with the light microscope, increases the opacity of the cells to electrons and reduces intracellular differentiation. Treatment of the cells with 0.02 per cent sodium bicarbonate solution in water for $2\frac{1}{4}$ hours seems to reduce the opacity of the cytoplasm without affecting that of the granules. The result is an increased intracellular differentiation (fig. 2), so that the form, position, and number of the granules can be studied in the majority of the cells. Treatment of the cells for 5 hours with 2 per cent, by volume, of nitric acid in water also brings out the granules (fig. 3), although one notices a beginning of dissolution. Cells treated with 10 per cent hydrochloric acid, for the same length of time, appear uniformly black (fig. 4), an effect which is probably due to the dissolution of the granules. Moreover, the pictures of acid-treated cells suggest that the acid-dissolved granular material does not readily diffuse out of the cell. The above results confirm those of Knaysi (1942a) on the solubility of those granules.

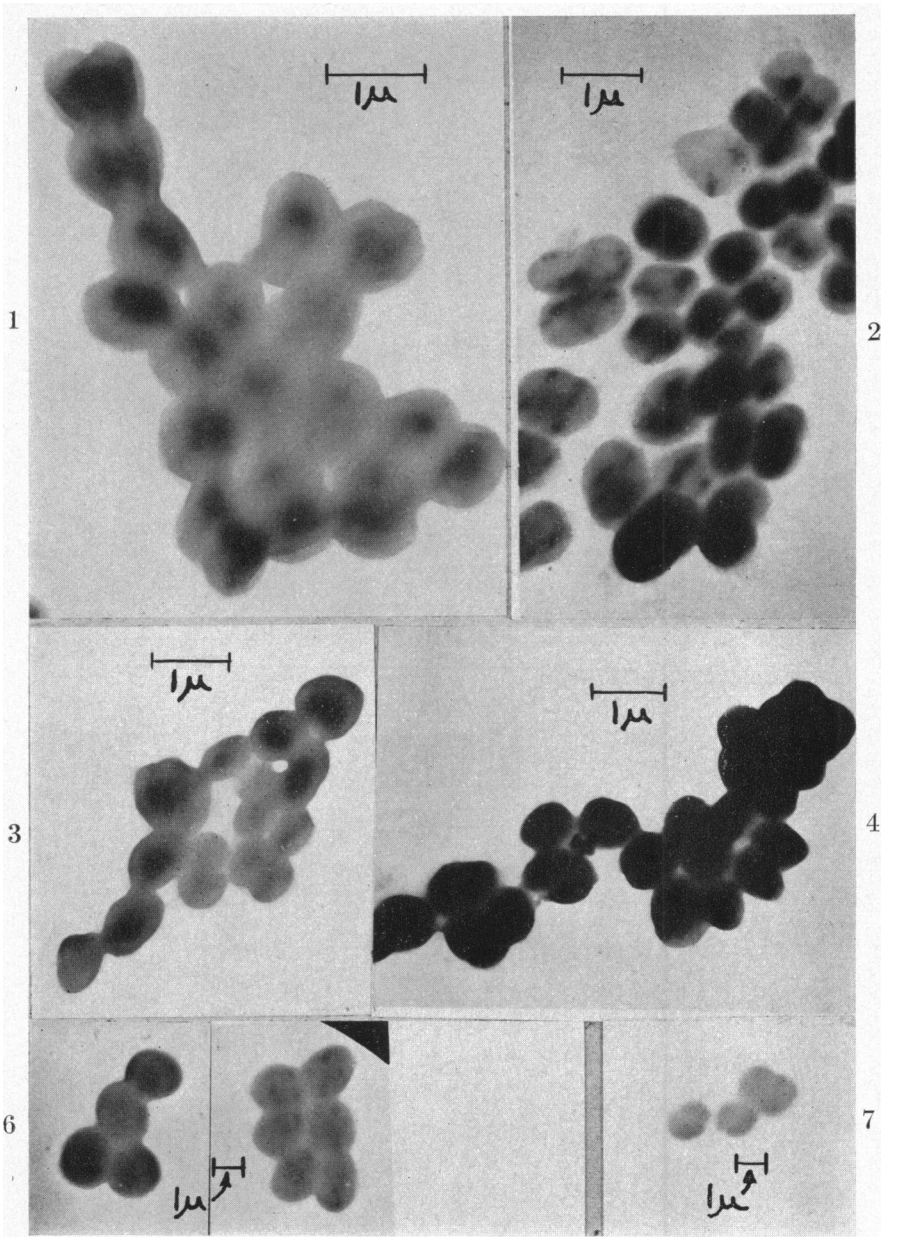

FIG. 1. S. *flavo-cyaneus*, 24 hrs. old at room temperature; meat infusion agar; no treatment; 60 kv. Magnification as reproduced × 13,000

FIG. 2. *S. flavo-cyaneus*, 48 hrs. old at room temperature; meat infusion agar; 2¼ hrs. in 0.02% $NaHCO_3$ in water; 60 kv. × 10,000

FIG. 3. Material as in fig. 2; 5 hrs. in 2% HNO_3 in water; 60 kv. × 10,000

FIG. 4. Material as in fig. 2; 5 hrs. in 10% HCl in water; 60 kv. × 10,000

FIG. 6. *S. flavo-cyaneus*, 14 hrs. old at room temperature; meat infusion agar; the culture was transferred twice before at a few hrs. intervals at 37°C; no treatment; 200 kv. × 4,000.

FIG. 7. *S. flavo-cyaneus*, 6 hrs. old at 37°C; meat infusion agar; the culture was inoculated with cells kept young by frequent transferring; treated with 0.04% $NaHCO_3$ for 1½ hrs; 200 kv. × 4,000.

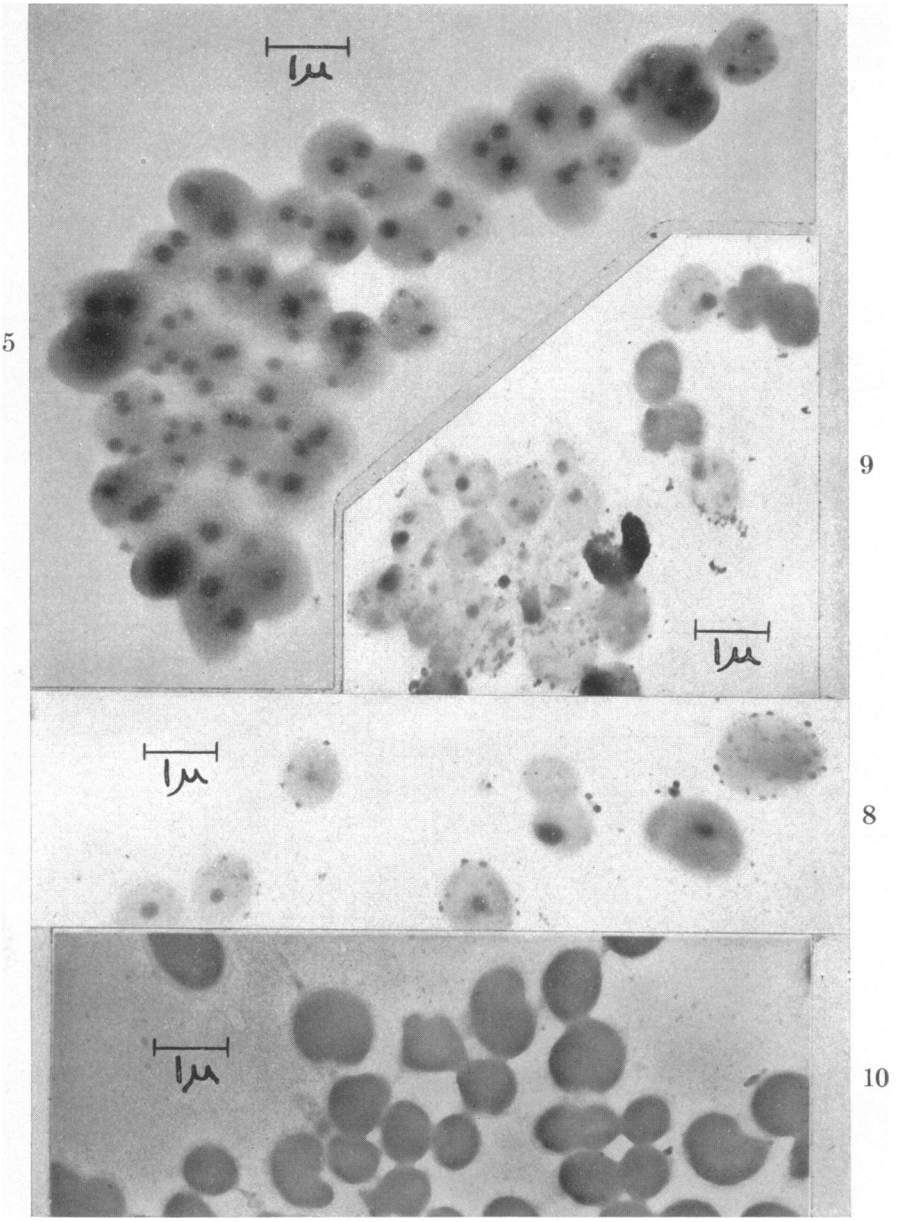

FIG. 5. Material as in fig. 2; no treatment; 127 kv; × 11,000
FIG. 8. *Neisseria meningitidis*, 22 hrs. old at 37°C. on 5% horse blood agar. Slide technique; heat fixed but otherwise untreated; 60 kv. × 10,000
FIG. 9. Material as in fig. 8; heat fixed and exposed to distilled water at 80°C. for 10 minutes; 60 kv. × 10,000
FIG. 10. *Neisseria gonorrhoeae*, strain P96A; 24 hrs. old at 37°C.; chocolate agar; no treatment; 60 kv. × 10,000

Figures 5 to 7 were taken at the high voltages indicated in the legend. Figure 5 represents cells taken from a 48-hour-old culture grown on meat infusion agar at room temperature. It shows a majority of ellipsoidal cells containing two

granules each. The granules range from 40 to 360 mμ in diameter. Some of those granules appear to be undergoing division. It can also be seen that an occasional cell contains a single granule sometimes in the process of division, while a number of cells contain 3, 4, or 5 granules. As a rule, when a cell contains several granules, these are somewhat reduced in size. Correlation of the number of granules with the state of the cell is not possible because, at the high voltages used, the electron microscope apparently fails to record the early stages of division; this can also be deduced from a comparison between figures 6 and 7. In both cases, the cells were taken from very young, actively growing cultures. The cells of figure 6 were untreated, while those of figure 7 were placed in a solution of 0.04 per cent sodium bicarbonate for 1½ hour. Treatment with bicarbonate seems to bring out the boundaries of the forming sister cells. Cells of *S. flavo-cyaneus* have been disintegrated by sonic vibration and the resultant material passed through a Seitz filter. The filtrate was dialyzed and dried from the frozen state as described earlier by Sevag, Smolens and Stern (1941). After throwing down a sediment in the angle centrifuge, the supernatant was spun in the ultracentrifuge and pellets were obtained consisting of material which appeared granular in electron micrographs. Their particle size, although on the average somewhat smaller than the intracellular granules in fig. 5, were of the same order of magnitude; they gave a positive Feulgen reaction for desoxyribose nucleic acid and chemical analysis showed purine nitrogen and phosphorus to be present. These granules stained with carbol thionine and, like the intracellular granules, retained their color after treatment with 0.15 per cent acetic acid.

Neisseria meningitidis. Figures 8 and 9, both taken at 60 kv., represent cells from a 22-hour-old culture on horse blood agar at 37°C. The cells of figure 8 were untreated, while those of figure 9 were exposed to the action of distilled water at 80°C. for 10 minutes. The cells contain each a single granule which is not soluble in hot water and, therefore, is not volutin. Further studies may prove that those granules are of nuclear nature.

Neisseria gonorrhoeae, *Staphylococcus aureus*, and *Streptococcus pyogenes*, strains 1048M and C203M. Pictures of these organisms, taken at 60 kilovolts, although showing the usual differentiation of cell-wall and inner protoplasm, reveal no internal structure, (figs. 10–12). Various treatments of the cells of *Staphylococcus aureus*, including treatment with bicarbonate, failed to alter the situation. Observed at high voltages, the cells of this group of bacteria become sufficiently transparent, but no intracellular differentiation has been observed by us either in fairly young or in mature cultures (figs. 13–15).

Brucella abortus and *Pasteurella pestis.* *Brucella abortus* (figs. 16–18) was studied from cultures 2, 14½, and 48 hours old at 37°C. using the 60 kilovolt microscope. The cells of the 2-hour-old culture either appear homogeneous or show diffuse, transparent areas. Cells taken from the 14½-hour-old culture show one or two clearly defined, transparent areas with clear cut margins, while the 48-hour-old culture gives cells occasionally showing internal granules. It is therefore possible that we are dealing here with vacuoles which become more opaque as they contain more and more reserve material. We believe this con-

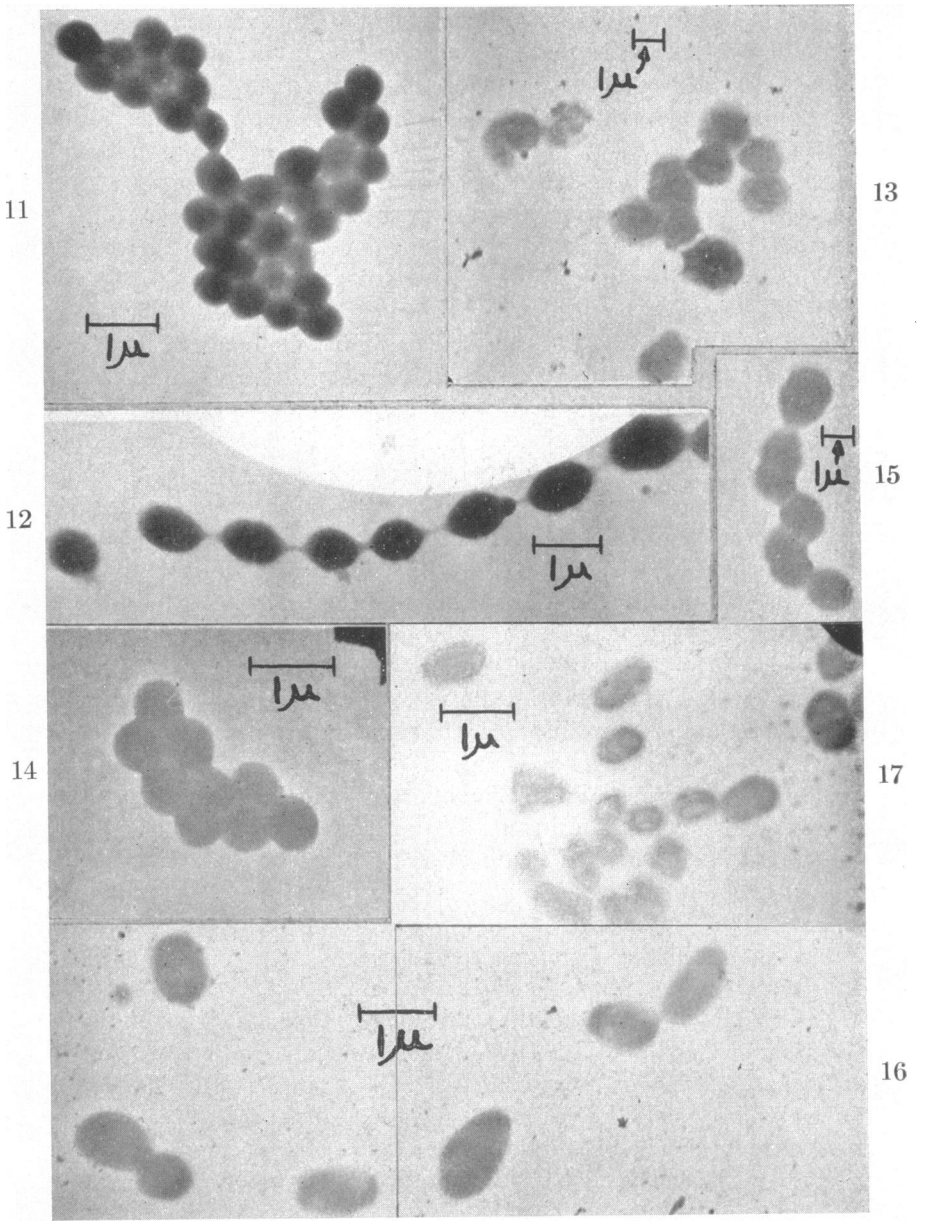

FIG. 11. *S. aureus*, strain P76; 22 hrs. old at 37°C.; meat infusion agar; 60 kv. × 9,300
FIG. 12. *S. pyogenes*, strain C203M; 6 days old at room temperature; 5% horse blood infusion agar; suspension made with 0.85% NaCl; salt subsequently removed; 60 kv. × 9,300.
FIG. 13. *Neisseria gonorrhoeae*, 23 hrs. old at 37°C. plus 7 hrs. at room temperature; 5% horse blood infusion agar; 200 kv. × 4,000
FIG. 14. *Staphylococcus aureus*, 24 hrs. old at 37°C.; no treatment; 200 kv. × 11,000
FIG. 15. *S. pyogenes*, strain 1048M; 15½ hrs. old at 37°C. plus 8 hrs. at room temperature; 5% horse blood infusion agar; 200 kv. × 4,000
FIG. 16. *Brucella abortus* var. *suis* 2 hrs. old at 37°C.; meat infusion agar; inoculated from a young culture. Slide technique; heat fixed; otherwise untreated; 60 kv. × 10,000
FIG. 17. *Brucella abortus* var. *suis* 14½ hrs. old at 37°C.; meat infusion agar; technique as in fig. 16; 60 kv. × 10,000

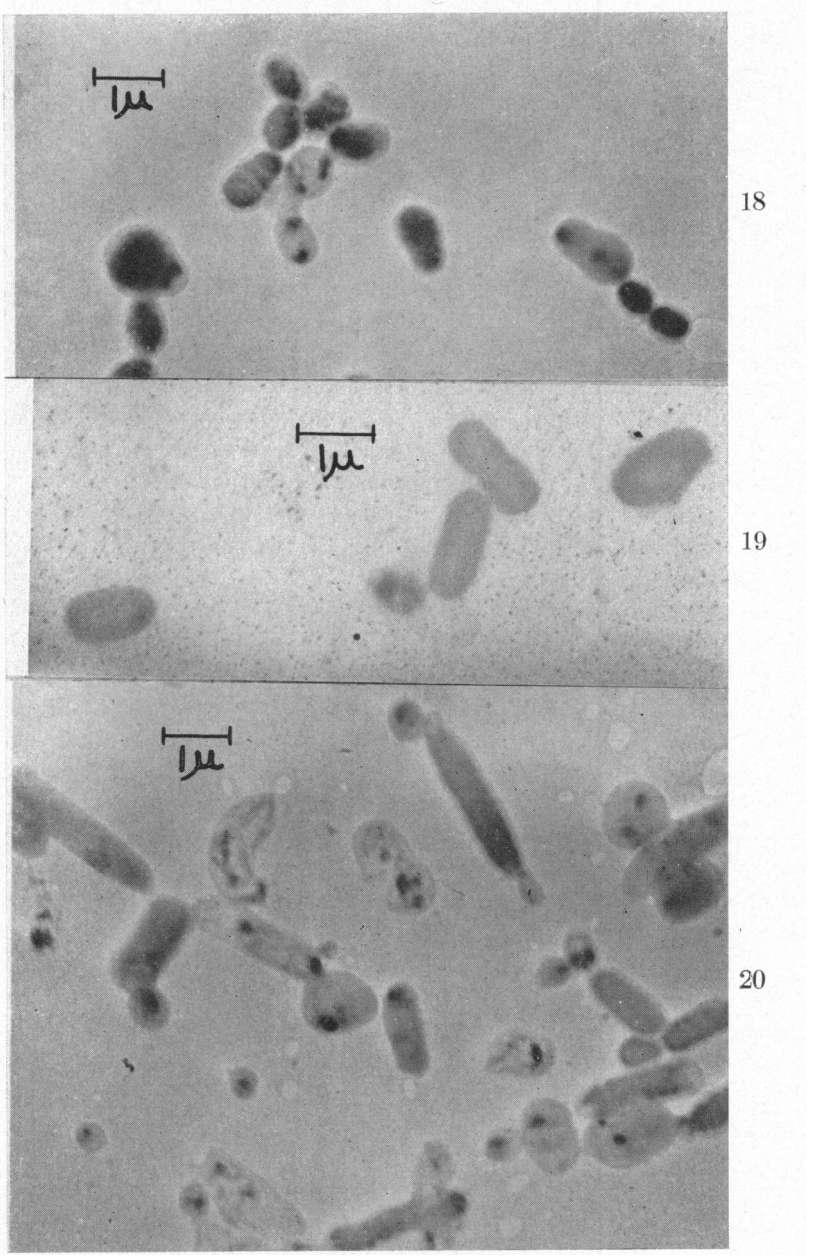

FIG. 18. *Brucella abortus*, strain P62; var. *bovis* 48 hrs. old at 37°C.; 5% horse blood infusion agar; 60 kv. × 9,300

FIG. 19. *Pasteurella pestis*, strain 87, avirulent; 2 hrs. old at 37°C.; meat infusion agar; inoculated from a young culture; slide technique; heat fixed, otherwise untreated; 60 kv. × 10,000.

FIG. 20. *Pasteurella pestis*, strain 87, avirulent; 22 hrs. old at 37°C. plus 2 days at room temperature; standard technique (a); 60 kv. × 9,300

clusion to be correct, although the 48-hour-old culture was of a different strain and grown on a different medium. Similar observations on *Pasteurella pestis* lead to the same conclusion (figs. 19 and 20). In both cases, treatment of the

young cells with boiling water causes extensive shrinkage of the protoplasm and the appearance of internal granules unobserved in untreated cells, and which should be considered as artifacts, probably representing particles of coagulated protein.

DISCUSSION

The observations on *Staphylococcus flavo-cyaneus* reported in this paper can be summarized as follows: The cells of that microorganism may contain one or several granules which are insoluble in 0.02 per cent sodium bicarbonate or in 2 per cent nitric acid, but which dissolve in 10 per cent hydrochloric acid within the time limits indicated in the text. They often appear paired or constricted, indicating a probable division, for the probability that two granules lying side by side inside of a sphere should occupy that position accidentally can be calculated (Borel and Deltheil, 1926) and in the present case it is about one in one thousand. On the basis of their morphology, position and solubility, we believe that our granules are identical with those studied cytologically and microchemically by Knaysi (1942a), whose results indicate that pairing and constriction occur only in growing, not in resting cells.

In very young, actively growing cells, the granules are reduced in size and in some cells are not visible (figs. 6 and 7). One also notices a certain increase in the opacity of the cytoplasm. Those observations can readily be explained by assuming that, during active growth, the material forming granules exists partially in solution. As to the significance of the intracellular granules of *Staphylococcus flavo-cyaneus*, we believe that the present investigation supports the evidence brought out by Knaysi that they represent the cell nuclei.

Of special interest are the results of our study of the cells of *Neisseria gonorrhoeae*, *Staphylococcus aureus*, and *Streptococcus pyogenes*, in the cells of which we were unable to detect any internal structure. At first we were inclined to explain that fact by the opacity of the cells of those organisms. However, the same results were obtained when observations were made at high voltages and the cells became sufficiently transparent.[1] There are, therefore, two possible conclusions to be drawn from such observations, the first is to say that the cells of those organisms contain no nuclear material, and the second is to say that they contain nuclear material in solution, or so dispersed that it is not revealed by the electron microscope. Analytical determinations of nucleic acid in the cells of *Streptococcus pyogenes*, 1048M and C203M, by Sevag, Smolens, and Lackman (1940), in particular the demonstration of thymonucleic acid, rule out the first conclusion and justify the second. The above investigators reported that, based on their dry weight, strain 1048M contains 20.4 per cent and strain C203M contains 20.7 per cent nucleic acid, 10 to 30 per cent of which is of the desoxyribose type. These figures must correspond to the surprisingly high nucleoprotein content of the streptococcal cell, and recall an interesting hypothesis advanced by the late

[1] Although heavy particles containing d-ribose nucleoprotein have been isolated from Streptococcus pyogenes by Sevag, Smolens and Stern (1941), these are too small (mean diameter = 42 mμ) to be resolved when within the bacterial protoplasm

Sir Patrick Laidlaw (1938). According to that hypothesis, the viruses may have undergone a reduction in size through parasitism, losing more and more of the essential factors of growth and multiplication until, at the end of the scale, the smallest viruses would have retained only the substance that transmits the characters of the species, namely nucleoprotein.[2] Whether there is any significance in the fact that viruses of the tobacco mosaic type contain exclusively the d-ribose type of nucleic acid, (Allen, 1941, and Cohen and Stanley, 1942), cannot at the present be said.

The absence of a granular nucleus in the cells of a strain of *Neisseria gonorrhoeae* was surprising to us, first because of the close relation between this organism and of a strain of *Neisseria meningitidis* in which an intracellular granule, possibly of nuclear nature, has been demonstrated by us, and second because of the claim made by Schumacher (1926) of having demonstrated a nucleus in the cell of the gonococcus. Therefore, if Schumacher's claim is correct, we are led to explain the discrepancy on the basis of a difference in the strains.

It appears, therefore, from the present investigation, that failure, in the past, to demonstrate a nucleus in the cell of bacteria has not always been due to small size. It seems that bacteria are not homogeneous with respect to their nuclear condition, and that in certain bacteria a change in the state of the nuclear material with the development of the cell has been demonstrated. This may explain the well-known fluidity of bacterial characteristics and reconcile numerous discrepancies found in the literature. We believe that the status of the problem is now such that it becomes unnecessary to hold that the bacterial cell has no nucleus, or that the bacterial nucleus is too small to be seen. Moreover, we believe to have brought out evidence which renders it meaningless to speak of a granular nucleus, diffuse nucleus, and central body as distinct hypotheses advanced to describe the state of the nuclear material in bacteria. As to the remaining hypothesis that the whole bacterial cell is the equivalent of a nucleus, we consider it too subjective to merit consideration. Experimentally, it is based on the same type of evidence that supports the hypothesis of a diffuse nucleus.

Although the views that the nuclear condition in different bacteria may be different, and that the state of the nuclear material in the same bacteria may change, are not new (Guilliermond, 1908, Dobell, 1911, Douglas and Distaso, 1912), yet we hope that the present investigation, by using new and powerful instruments of observation, and by making use of certain organisms that have been adequately studied cytologically or chemically will have contributed toward placing the problem of the bacterial nucleus in the objective domain of scientific research.

We wish to express appreciation to Dr. V. K. Zworykin for the privilege of using the high voltage electron microscope and for the courtesies of the RCA Research Laboratories; also to Dr. Thomas F. Anderson and Dr. R. S. Baker for taking the pictures. We would thank Dr. Harry E. Morton, also, for aid

[2] The alternative possibility, that viruses may be primordial rather than degenerate forms, must not be lost sight of, however.

with the cultures. For aid in the preparation and study of the extracellular granules from *S. flavo-cyaneus* we are indebted to Dr. M. G. Sevag, Dr. Leslie A. Chambers, Mr. F. Heinmets and Mr. J. Smolens.

SUMMARY

The structure of a number of bacteria has been studied with the electron microscope. Organisms opaque to electrons at 60 kilovolts were also observed at higher voltages.

The cell of *Staphylococcus flavo-cyaneus* contains one or more granules having similar solubilities to nucleoproteins and which often appear constricted or in pairs. In very young, actively-growing cells, the granules demonstrable at high voltages are reduced in size and there is evidence that the nuclear material is then partially in solution. A strain of *Neisseria meningitidis* also shows granules which are insoluble in hot water and which are likely to be of nuclear nature.

On the other hand, the cells of strains of *Neisseria gonorrhoeae*, *Staphylococcus aureus*, and *Streptococcus pyogenes*, strains 1048M and C203M, appear homogeneous at all voltages. Thymonucleic acid having been demonstrated by other investigators in these strains of *Streptococcus pyogenes*, it is probable that their cells contain the nuclear material in solution or very fine dispersion.

The cells of *Brucella abortus* and *Pasteurella pestis* appear uniform or show transparent areas in very young, actively growing cultures. Gradually, one or two transparent areas are seen which we believe later become opaque. It is probable that we are here dealing with vacuoles and reserve material.

The results of the present investigation support the view that different bacteria may contain nuclear material in different states, and that the state of the nuclear material may change with the development of the cell.

BIBLIOGRAPHY

ALLEN, F. W. 1941 The biochemistry of the nucleic acids, purines, and pyrimidines. Ann. Rev. Biochem., x, 221–244.

BOREL, E., AND DELTHEIL, R. 1926 Probabilités, erreurs. Armand Colin, Paris, 2nd ed.

COHEN, S. S., AND STANLEY, W. M. 1942 The molecular size and shape of the nuclei acid of tobacco mosaic virus. J. Biol. Chem., **144,** 589–598.

DELAPORTE, B. 1939–1940 Recherches cytologiques sur les bactéries et les cyanophycées. Rev. gén. botan., **51,** 615–643, 689–708, 748–768; **52,** 40–48, 75–96.

DOBELL, C. 1911 Contributions to the cytology of bacteria. Quart. j. microscop. Sci., n.s., **56,** 395–506.

DOUGLAS, S. R., AND DISTASO, A. 1912 Etudes sur le noyau des bactéries. 1. Mem. sur un nouveau bacille dont le noyau est très évident. Zentr. Bakt. Parasitenk. I, Orig., **63,** 1–7.

FEULGEN, R., AND ROSSENBECK, H. 1924 Mikroskopisch-chemischer Nachweis einer Nucleinsäure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. Z. physiol. Chem., **135,** 203–248.

GUILLIERMOND, A. 1908 Contribution à l'étude cytologique des bacilles endosporés. Arch. Protistenk., **12,** 9–43.

HOLWECK, F. 1929 Production de rayons X monochromatiques de grande longueur d'onde. Action quantique sur les microbes. Compt. rend., **188,** 197–199.

KNAYSI, G. 1938 Cytology of bacteria. Botan. Rev., **4,** 83–112.

KNAYSI, G. 1941 A morphological study of *Streptococcus fecalis*. J. Bact., **42**, 575–586.

KNAYSI, G. 1942a The demonstration of a nucleus in the cell of a staphylococcus. J. Bact., **43**, 365–386.

KNAYSI, G. 1942b Unpublished.

KRUIS, K. 1913 Mikrophotographien der Strukturen lebender Organismen, insbesondere der Bakterienkerne mit ultraviolettem Licht. Česká Akademie věd a uměni v Praze. Bull. intern. acad. polon. sci., **18**, 141–160.

LACASSAGNE, A. 1929 Action des rayons X de grande longueur d'onde sur les microbes. Etablissement de statistiques précise de la mortalité des bactéries irradiées. Comp. rend., **188**, 200–202.

LAIDLAW, P. 1938 Virus diseases and viruses. The Rede Lecture. Cambridge University Press.

LEWIS, I. M. 1941 The cytology of bacteria. Bact. Revs., **5**, 181–230.

MARTON, L. 1941 The electron microscope. A new tool for bacteriological research. J. Bact., **41**, 397–413.

MUDD, S., AND ANDERSON, T. F. 1942 Unpublished data.

MUDD, S., AND LACKMAN, D. B. 1941 Bacterial morphology as shown by the electron microscope. I. Structural differentiation within the streptococcal cell. J. Bact., **41**, 415–420.

PIEKARSKI, G. 1939 Lichtoptische und übermikroskopische Untersuchungen zum Problem des Bakterienzellkerns. Zentr. Bakt. Parasitenk., I., Orig., **144**, 140–147.

SCHUMACHER, J. 1926 Ueber den Nachweis des Bakterienkerns und seine chemische Zusammensetzung. Zentr. Bakt. Parasitenk., I., Orig., **97**, 81–104.

SEVAG, M. G., SMOLENS, J., AND LACKMAN, D. B. 1940 The nucleic acid content and distribution in *Streptococcus pyogenes*. J. Biol. Chem., **134**, 523–529.

SEVAG, M. G., SMOLENS, J., AND STERN, K. G. 1941 Isolation and properties of pigmented heavy particles from Streptococcus pyogenés. J. Biol. Chem., **139**, 925–941.

ZWORYKIN, V. K., HILLIER, J., AND VANCE, A. W. 1941 A preliminary report on the development of a 300-kilovolt magnetic electron microscope. J. Applied Phys., **12**, 738–742.