ELECTRON AND LIGHT MICROSCOPIC STUDIES OF BACTERIAL NUCLEI

II. An Improved Staining Technique for the Nuclear Chromatin of Bacterial Cells[1]

ANDREW G. SMITH[2]

Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania

Received for publication January 25, 1950

When bacteria are stained by conventional procedures, the basophilia of the cytoplasm and cytoplasmic membrane obscure the nuclear chromatin. This cytoplasmic basophilia may be removed either by hydrochloric acid treatment, as in the procedure developed and widely applied by C. F. Robinow, or by treatment with ribonuclease, as discovered by the workers in Boivin's laboratory (Boivin, 1947; Tulasne and Vendrely, 1947*a*,*b*, 1948). Nuclear staining procedures for bacteria have been well reviewed by Robinow (1945) and more recently by Hillier, Mudd, and Smith (1949).

DeLamater (1948) and DeLamater, Mescon, and Barger (1950) have shown that the staining of nuclear chromatin of certain fungi, which ordinarily are colored weakly in the Feulgen reaction or by chromatin dyes, may be intensified by mordanting with formaldehyde. A procedure is described in the present paper which is believed to possess advantages in terms of sharpness of definition of nuclear chromatin, of keeping qualities, of simplicity, and of rapidity of preparation. This procedure is modified from the procedures of Robinow and of DeLamater.

MATERIALS AND METHODS

The organisms to be reported on in this paper are *Escherichia coli*, strain B, a strain of *Proteus* (labeled P-44), a strain of *Staphylococcus aureus* (labeled P-78), and *Bacillus megatherium*. Stock cultures of these organisms were maintained on nutrient agar slants under mineral oil (Morton and Pulaski, 1938) at room temperature. Subcultures from stock were made into the enriched tryptone glucose yeast extract broth described by Morton and Engley (1945), herein referred to as M and E medium. Cultures were incubated at 37 C.

The following technique for demonstrating the chromatinic structure of bacterial cells was found to be generally applicable. From an overnight broth culture of the organism to be studied, a dilute broth suspension is spread (with the aid of a glass spreader) on M and E medium solidified with 1.5 per cent agar;

[1] This research has been aided by a grant from the Damon Runyon Fund through the American Cancer Society as recommended by the Committee on Growth of the National Research Council.

[2] Present address: Department of Bacteriology, School of Medicine, University of Maryland, Baltimore 1, Maryland.

0.05 to 0.1 ml of a suspension of organisms numbering around 1.0×10^8 per ml is a sufficient volume for spreading on freshly made agar plates that have been partially dried through overnight incubation at 37 C. The freshly inoculated plate is then incubated until the cells are in an actively growing state and the surface of the agar plate contains confluent growth not more than a cell or so in thickness. The time for fulfilling this condition varies with different organisms. With rapidly growing organisms such as *E. coli* B, *Proteus*, etc., $1\frac{1}{2}$ hours usually is sufficient. When growth has reached the desired stage, an agar block is cut out (with the aid of a scalpel or agar knife) and the cells are fixed for 1 to 3 minutes in 2 per cent osmium tetraoxide vapor while still on the agar (Robinow, 1942, 1944). Following fixation, an impression film is made on a thin square cover glass. If the agar growth has become too thick for the making of satisfactory impression films, an expedient is to invert a rather narrow strip of the agar growth on a cover glass and push it evenly across its width. A satisfactory preparation can usually be made in this manner. Such films air-dry almost instantaneously.

The osmium-fixed film is next subjected to treatment in normal hydrochloric acid at 60 C. This is the most critical step in the process. The optimal time period for the acid treatment can only be arrived at through an extensive series of trials. It will vary for different organisms, a minute or two in this respect becoming extremely important owing to the size of the organisms and their depicted structures (DeLamater *et al.*, 1950). The period of acid treatment will also vary depending upon the environmental conditions; e.g., *E. coli* B when grown on solid medium has been found to have an optimal acid treatment in from 5 to 7 minutes; when grown in liquid medium the optimal acid treatment has been about 3 minutes. In addition to species differences and growth conditions, the phase of growth is important in determining the acid treatment period; e.g., *Proteus* P-44 when in the filamentous swarming phase may receive an optimal acid treatment in about 4 minutes; in the small cell and consolidation phases 1 or 2 minutes longer may be necessary. Filamentous cells of *E. coli* also have been found to respond more quickly to acid treatment than the smaller cells in that species.

Following hydrochloric acid treatment the preparation is washed in distilled water and then mordanted in 1 per cent formaldehyde for 2 to 4 minutes. Two per cent formaldehyde as used by DeLamater (1948) on fungi was found to increase the basophilia of the cytoplasm of bacterial cells to the extent of subtracting considerably from the differentiation of the chromatinic structure. The film is washed again in distilled water after the foregoing treatment and then stained in 0.3 per cent aqueous basic fuchsin for 15 to 30 seconds. This is followed once more by washing and the preparation is then mounted in tap water on an ordinary microslide; the excess water is absorbed with pieces of filter paper and the cover glass sealed to the slide with melted paraffin wax (applied with the aid of a camel's-hair brush).

Specimens prepared by this technique were observed by means of a Zeiss microscope having an aplanatic condenser with a N.A. = 1.40, an apochromatic 90 × oil immersion objective with a N.A. = 1.30, and either a 10 × or a 15

× compensating ocular. The source of critical illumination was a Bausch and Lomb research microlamp equipped with a ribbon filament bulb and transformer. In addition, a water cell holder and filter rack were attached. Conditions of optimal contrast and definition were obtained when Wratten "M" filters in a combination resulting in a dark green were used. Such a color is given by combining Wratten G filter (no. 15), deep yellow, with Wratten H filter (no. 45), deep blue. For maximal resolution it was of course necessary to use oil between the condenser and microslide.

RESULTS

Cultures for all of the pictures in figures 1 through 8 were grown at 37 C on M and E agar, fixed in osmium tetraoxide vapor, treated in N hydrochloric acid at 60 C, mordanted in formaldehyde, stained in basic fuchsin, mounted in tap water, and sealed with paraffin. Photography was either on "panatomic X" film or on Kodak "M" plate. Wratten G and B or G and H filters were used. The details of the several steps of processing are given under the individual figures.

Escherichia coli B. Figure 1 illustrates satisfactorily the fine, rodlike structure of the chromatinic bodies of *E. coli* B. Figure 2 shows the chromatinic bodies to be more nearly rounded up. This effect comes from overfixation in osmium tetraoxide vapor. The latter specimen was fixed for 3 minutes; the former for 1 minute. The apparent condensing and granulation of the chromatin, although resulting some from the general shrinking action of osmium tetraoxide on the bacterial cells, is felt to be due, in addition, to the subsequent effect of the hydrochloric acid treatment on these osmium-tetraoxide-fixed specimens. Insufficient data exist at this time for proving this contention, but further work is planned. According to McClung's *Microscopical Technique* (1937), osmium tetraoxide is used in the Kopsch method for fixing small tissue sections to demonstrate the Golgi apparatus; however, considerable shrinkage and brittleness result. Osmium tetraoxide has rather poor penetrating power when used on tissue specimens and has a tendency to overfix on the surface and underfix internally. With specimens the size of bacteria this differential action probably is lost and the possibility exists of overfixing the whole bacterial protoplasm. This danger becomes more real when it is noted that osmium tetraoxide is more highly penetrating in vapor than in solution (Lee, 1937) and in certain cytological techniques may cause distortion and shrinkage. In addition, it is noted that secondary changes are known to take place in specimens that are well fixed in osmium when they are placed in watery media at elevated temperatures (40 to 60 C). That overfixation in osmium tetraoxide does cause secondary changes to take place is corroborated by a comparison of figures 1 and 2. The danger of overfixation is greater when the vapors from a fresh quantity of osmium tetraoxide are used.

The structures depicted in figure 1 are believed to represent more closely the natural distribution of chromatin in the bacterial cell than the structures in figure 2 for additional reasons. Those in figure 1 are morphologically analogous to what is obtained in ribonuclease-digested preparations of *E. coli* B, which have been mordanted with formaldehyde and stained with basic fuchsin. In such

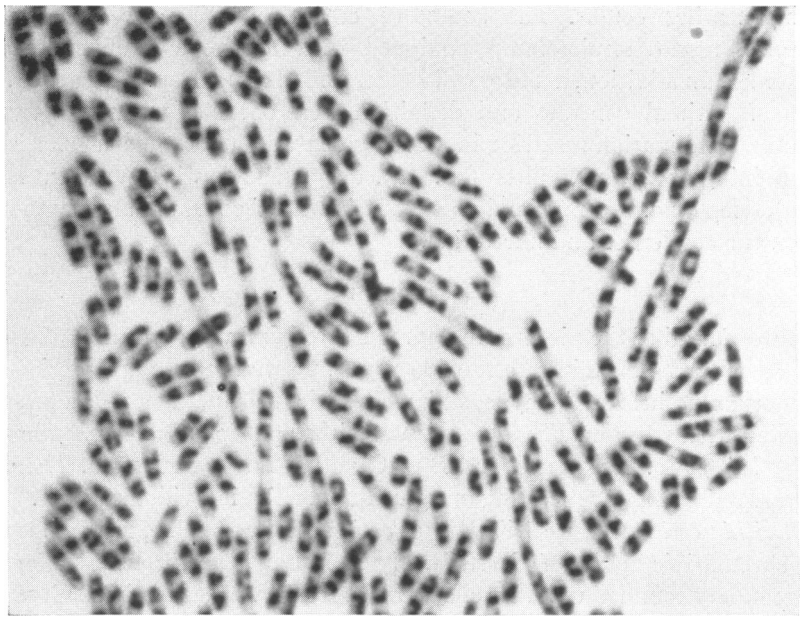

Figure 1. Escherichia coli B. Growth 1¼ hours; fixed 1 minute; hydrochloric acid 6 minutes; formaldehyde 3 minutes; basic fuchsin 30 seconds. 2,500 ×. Photography: "panatomic X" film with G and B filters; 5 seconds' exposure.

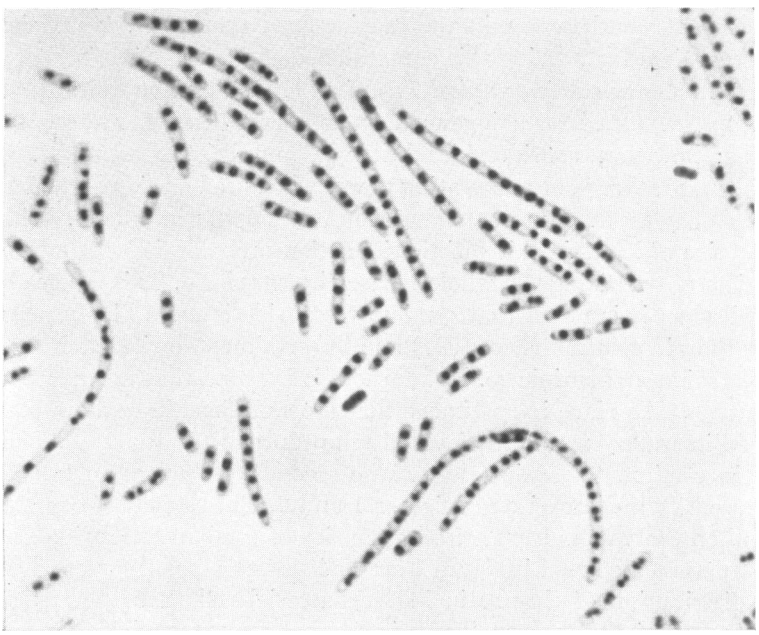

Figure 2. Escherichia coli B. Growth 1½ hours; fixed 3 minutes; hydrochloric acid 7 minutes; formaldehyde 3 minutes; basic fuchsin 30 seconds. 2,400 ×. Photography: Kodak "M" plate with G and H filters; 70 seconds' exposure.

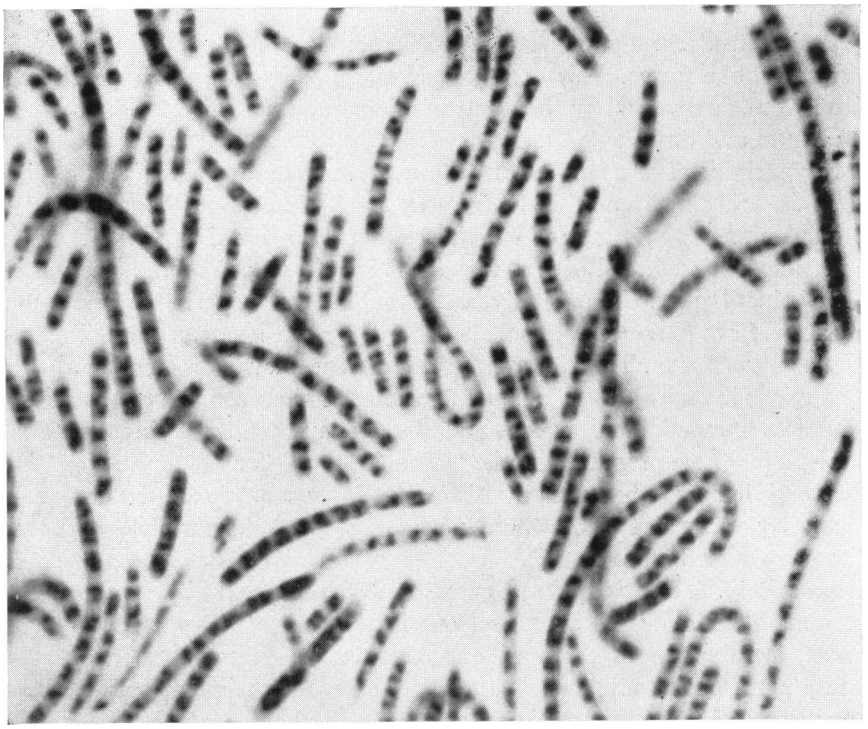

Figure 3. Proteus P–44. Organism passing from swarming into consolidation stage; fixed 1 minute; hydrochloric acid 5 minutes; formaldehyde 3 minutes; basic fuchsin 30 seconds. 2,500 ×. Photography: "panatomic X" film with G and B filters; 4 seconds' exposure.

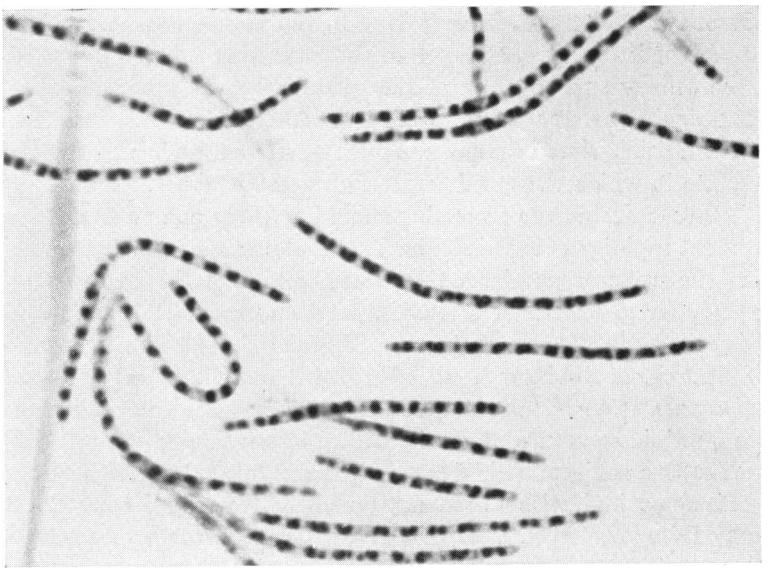

Figure 4. Proteus P–44. Organism in the swarming stage; fixed 3 minutes; hydrochloric acid 5 minutes; formaldehyde 3 minutes; basic fuchsin 20 seconds. 2,500 ×. Photography: Kodak "M" plate with G and H filters; 70 seconds' exposure.

preparations the chromatinic structures extend completely across the cell in apparent rodlike forms. This is also their morphology, whether in the fixed or unfixed state, as revealed by the electron microscope (Hillier, Mudd, and Smith, 1949; Mudd and Smith, 1950).

A very striking revelation in figures 1 and 2, evident after a little study, is the distinct doubling of chromatinic bodies coincident with, but prior to, cell division. There are numerous examples in both fields of cells containing 2, 4, 8, and 16 chromatinic bodies. In cultures of this age it is not unusual to find cells containing 32 distinct bodies. Undoubtedly, an example of this exists in the large, curved filamentous form of figure 2, but a distortion near the center of the cell prevents absolute enumeration. The various stages in this geometric progression are much more obvious in figure 1 than in figure 2, where the chromatin has condensed into more or less rounded, homogeneous granules. In young cultures under the growth conditions for these preparations the "trinucleate cell" or cell containing 3 pairs of chromatinic bodies described by Bisset (1948*a*) was not found with any degree of regularity or certainty, and where suspected might well be explained as a stage in the process of doubling.

Proteus P-44. A comparison of figure 3 with figure 4 will illustrate for *Proteus* P-44 what has already been pointed out in the case of *E. coli* B, namely, the effect of overfixation in osmium tetraoxide vapor. Figure 3 was fixed for 1 minute; figure 4 for 3 minutes. In the latter case, the shrinkage of the cells and the tendency of the chromatinic structures to condense and round up is quite obvious.

The preparation in figure 4 was made from an impression film of the edge of a colony that was in the swarming stage. The doubling of chromatinic bodies, which was shown to be a striking feature in the filamentous cells of *E. coli* B, appears to be of irregular occurrence in the swarming cells of *Proteus* P-44. In figure 3 the cells are passing out of the swarming stage into the consolidation stage. Here again the doubling process is irregular. However, this irregularity may be a condition that is more apparent than real owing to the extremely dynamic state in which these cells exist during these stages. Microscopic observation of *Proteus* during the swarming stage impresses one profoundly with the apparent great rapidity with which new bacterial protoplasm is being anabolized. It has been the author's experience that when *Proteus* exists in the nonfilamentous stage the regular geometric relationship between chromatinic bodies in 1-, 2-, and 4-unit cells appears to occur just as it does in *E. coli*. This is borne out also in the publications of Robinow (1942, 1944, 1945) and Klieneberger-Nobel (1947).

Staphylococcus aureus P-78. A photographically satisfactory demonstration of the chromatinic structure of staphylococci in relation to the cell outline has proved difficult to achieve. This derives primarily from the fact that the form of the coccus is ellipsoidal, with a tendency during active growth toward a spherical morphology (Knaysi, 1944). Figures 5 and 6 show a variety of chromatinic arrangements, and in most cases these are defined with respect to the cell outline. The structures seen agree with respect to apparent size, number, and distribution, with the Feulgen-positive bodies demonstrated for staphylococci by Knaysi

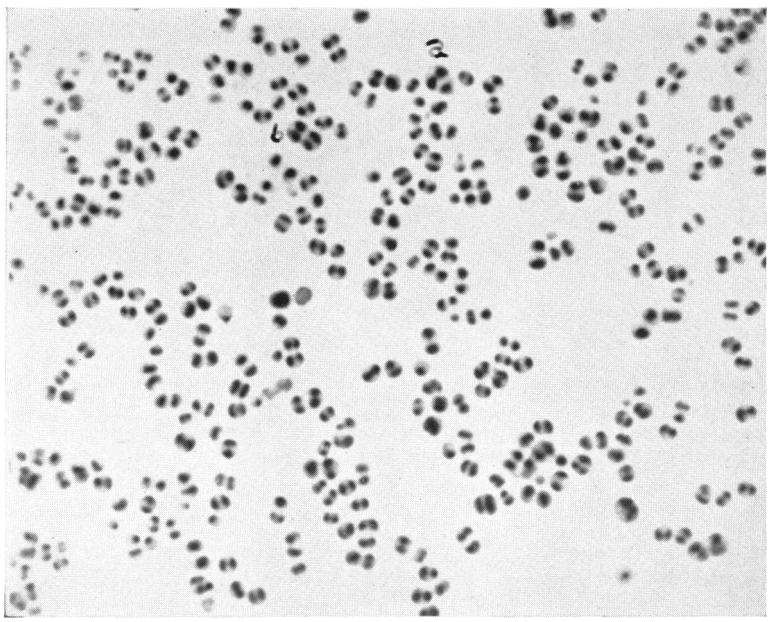

Figure 5. Staphylococcus aureus P–78. Growth 3½ hours; fixed 3 minutes; hydrochloric acid 5 minutes; formaldehyde 3 minutes; basic fuchsin 20 seconds. 2,400 ×. Photography: Kodak "M" plate with G and H filters; 70 seconds' exposure.

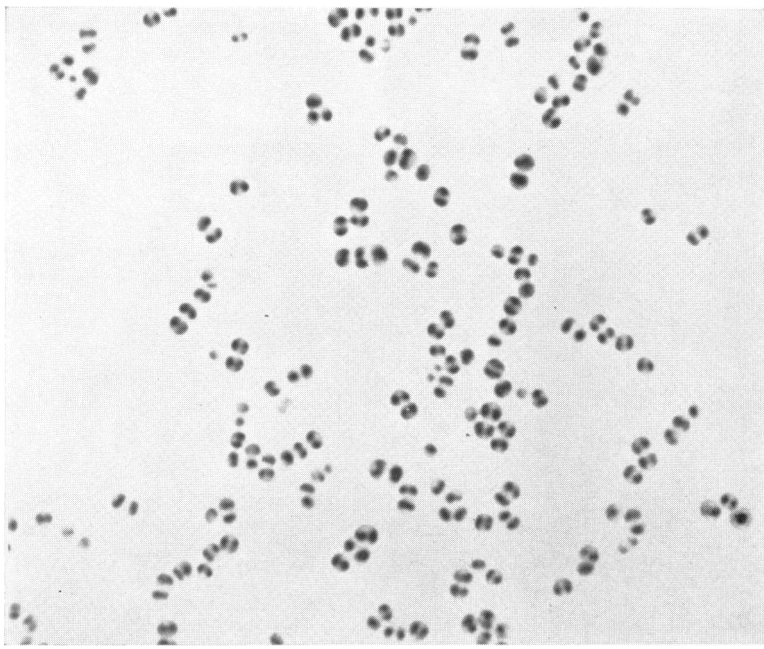

Figure 6. Staphylococcus aureus P–78. Same specimen as figure 5, taken 24 hours later. Specimen, mounted in slightly acid tap water, appeared to have differentiated somewhat upon standing.

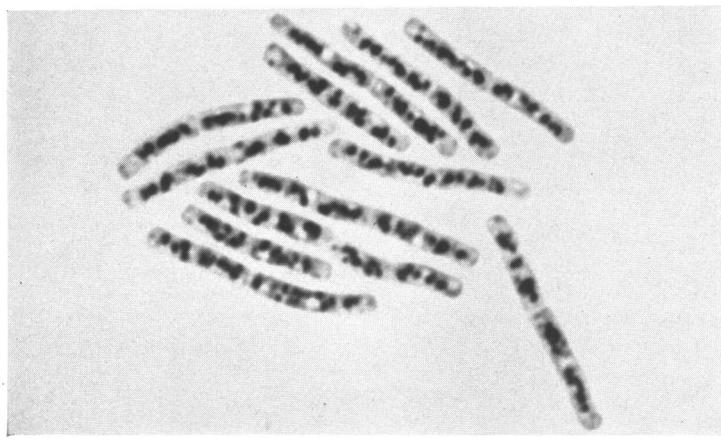

Figure 7. Bacillus megatherium. Growth 2½ hours; fixed 3 minutes; hydrochloric acid 5 minutes; formaldehyde 3 minutes; basic fuchsin 20 seconds. 2,500 ×. Photography: Kodak "M" plate with G and H filters; 70 seconds' exposure.

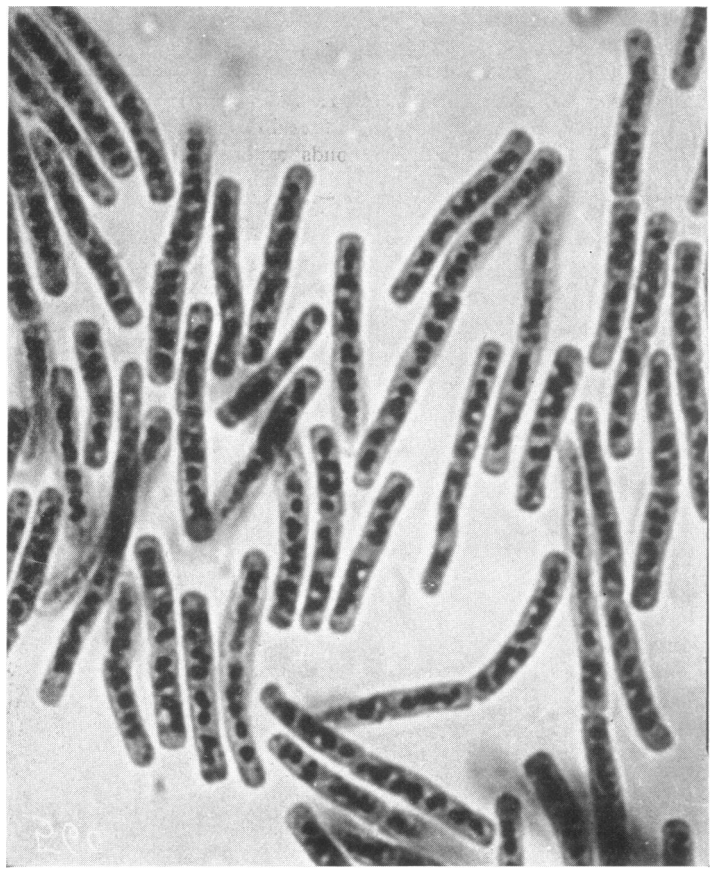

Figure 8. Bacillus megatherium. Growth 3 hours; fixed 3 minutes; hydrochloric acid 5 minutes; formaldehyde 3 minutes; basic fuchsin 30 seconds. 3,300 ×. Photography: "panatomic X" film with G and H filters; 6 minutes' exposure.

(1942). Robinow (1942) demonstrated Feulgen-positive bodies in two species of *Sarcina*. The distribution of chromatin in the foregoing figures is analogous, also, to the Feulgen-positive material depicted in the cellular units of Robinow's *Sarcina* packets.

In the figures one may see cells with but a single, round granule of chromatin. These forms appear to be in the minority. In the majority of cases, there appears to be a distinct pair of chromatinic structures associated with the dividing cell, and their morphology seems to vary with the stage of division; hence, in what might be an early phase of cell division, one can see two distinct half-moon basophilic bodies separated by a rather narrow nonbasophilic region. Cells further along in division show pairs of structures that are more rounded up and in which the volume of basophilic material has apparently decreased, compared to the earlier structures described above. Often these paired structures are connected by a strand of chromatin. There are examples present that suggest that, once division is complete, the new cells start off with a broad strand of chromatin directly through the center of each cell, and this immediately begins to constrict in preparation for a new cycle of karyokinesis.

At points *a* and *b* in figure 5 there are examples in which apparently karyokinesis has progressed more rapidly than cytokinesis. Such formations are not uncommonly found in young actively growing cultures, and no doubt they are analogous to what one finds in the rodlike organisms in which the rate of division and distribution of chromatinic structures has progressed much more rapidly than cell division per se. In excellent ultraviolet light micrographs, Smiles, Welch, and Elford (1948) show forms analogous to that at *a*. The existence of these trisegmented cells they attributed to the action of penicillin and showed at which stage the growth and development of the cell was arrested by the drug. They showed no tetrasegmented cocci as at *b*.

Bisset (1948*b*) has investigated the chromatinic structure of gram-positive coccus forms using the conventional Robinow technique and of gram-negative coccus forms (1948*c*) using the methylene blue eosin technique of Badian (1933). According to Bisset the gram-negative cocci do not respond well to the acid Giemsa technique. His results upon applying Badian's technique to a gram-negative coccus isolated from feces are excellent. Bisset's work on gram-positive cocci included both streptococci and staphylococci. Concerning the former, cocci of the long-chained strains were found to be divided frequently by transverse septa into two cells, each containing a single chromatinic body. Short-chained strains failed to form transverse septa, and the cocci divided by constriction. Among staphylococci, septate and nonseptate forms were found also. In the nonseptate form only a single granule appeared to be present, central or eccentric in position. In the septate form the picture was very much the same as presented in figures 5 and 6, even to the extent of showing trisegmented cells. The nonseptate *Staphylococcus* was less commonly found, but it occurred in strains of *S. albus* and *S. aureus*, and morphologically the chromatinic structure was similar to that in a strain of *Sarcina lutea* examined. The most recent application of Robinow's technique to a coccus form is to be found in the work of Johnson and

Gray (1949). Here young cells of *Photobacterium phosphoreum*, a psychrophilic marine luminous bacterium, were shown to give chromatinic structures quite analogous to those shown in figures 5 and 6.

In studies on the cytology of staphylococci using ribonuclease to destroy the basophilia of the cytoplasmic constituents, Tulasne and Vendrely (1948) describe and illustrate results that agree well with those presented in this paper. They compared cells grown normally with those grown under the influence of sublethal concentrations of penicillin. In the normal cells, 1 and 2 nuclear structures were normally found; rarely 3 and 4. Under the influence of penicillin, cytokinesis was halted, much swelling took place, and 2 to 4 and sometimes 5 nuclear structures were demonstrated. Destruction of the penicillin caused the cells to return to a normal cytokinesis cycle.

Bacillus megatherium. Of the four species presented in this paper, the chromatinic structure of *B. megatherium* appears to be the most complicated and hardest to interpret. The chromatin does not appear to be distributed with the regularity that is associated with like structures in *E. coli* B and *Proteus* P-44; rather it is distributed more often as groups of granular masses or as strands of the same. Individual forms of the chromatinic bodies range from spherical through dumbbell-shaped to definite rodlets. The problem of overfixation was not studied in the cases of the two species *S. aureus* and *B. megatherium*, so the question as to whether these various chromatinic arrangements are the result of such a condition cannot be answered at this time. Future work is to be done on this. According to Robinow (1942) acid Giemsa preparations of *B. megatherium* are "difficult to differentiate satisfactorily." He found the Feulgen technique to give the best results, showing the chromatinic structures to be essentially the same as in other aerobic sporeformers.

An interesting structure that became known as a result of applying the technique described in this paper is the vacuole that stands out so clearly in nearly every cellular unit in figure 7. They are identifiable, but not quite so readily, in some of the cellular units in figure 8. Associated with the vacuole in every case is chromatin; sometimes as a distinct granule, sometimes as several granules, and other times as strands of chromatin streaming away from opposite poles. Flewett (1948) discusses the presence of vacuoles in *Bacillus anthracis* that separate pairs of chromatinic bodies.

DISCUSSION

It is concluded that bacterial specimens treated in the manner prescribed in the text will present a truer definition of chromatin distribution than the same specimens treated by the conventional hydrochloric acid Giemsa and other techniques. Giemsa's stain, a rather complicated mixture of various thiazin-eosinates (Conn, 1946), is felt to be less selective in its staining reactions than basic fuchsin, a comparatively simple molecular entity when in pure form (pararosaniline). A comparison of Giemsa preparations with basic fuchsin preparations gives one the impression that chromatinic structures in the former are more often in the

form of unresolved pairs. Individual chromatinic structures stained by the mordanted basic fuchsin technique appear to be more delicate in structure than in corresponding Giemsa preparations.

The important contribution from the staining technique of DeLamater (1948) is the use of formaldehyde subsequent to hydrolysis but prior to staining. Just how formaldehyde reacts after hydrolysis to increase the affinity of nuclear components for basic fuchsin is not well understood. It is certain, however, that the stain becomes more tightly bound after this treatment than without it. Basic fuchsin preparations are excellent in themselves, but they fade rapidly. With the mordanting effect of formaldehyde they have been found to last up to three weeks in some cases without appreciable loss of intensity, which, incidentally, appears to be increased by the formaldehyde treatment. Attempts to make permanent mounts by the technique used by DeLamater for his fungous preparations have so far ended in failure. DeLamater, Mescon, and Barger (1950) critically discuss the DeLamater technique in relation to the Feulgen nucleal and periodic-Schiff reactions. In this they draw a possible parallel between the action here and that of formaldehyde in the Sørensen formaldehyde titration of acid radicals in amino acids, etc. If it acts to bind the amino groups in the desoxyribonucleoprotein molecule, then it would tend to increase the acidity of this component and, hence, its affinity for the *para*-amino groups of basic fuchsin.

The effect of overfixation in osmium tetraoxide vapor has been discussed with respect to the preparations presented in this paper. It may well be that in order to define more nearly the natural distribution of chromatin in the bacterial cell only an extremely small amount of fixation is necessary; perhaps fixation is not to be applied arbitrarily and indiscriminately, but to be determined more specifically for each species treated.

With respect to the period of treatment in hydrochloric acid, it has been the author's experience that one is much more likely to achieve a higher percentage of satisfactory preparations if, instead of adhering arbitrarily to a given time period empirically determined, one runs a series of 3 or 4 preparations through the hydrochloric acid bath with a minute between each preparation. If the organism responds well to the technique, usually at least one of the preparations will be outstanding with respect to brilliance of stain, contrast, and definition. As mentioned earlier, several factors are known to affect the response of the bacterial cell to the acid treatment; other unknown factors must exist.

No attempt has been made in this paper to interpret a nuclear cycle in the species studied (Bisset, 1949); nor has there been any attempt at further establishing the genetic significance of the chromatinic bodies demonstrated, although all who work with them are convinced they constitute the nuclear apparatus of the cells. It has been the objective to present a modified technique in bacterial cytology, which, it is felt, is simpler in application than other techniques existing for the same purpose, that of demonstrating the distribution of bacterial chromatin. In addition to its simplicity, preparations can be made with greater rapidity, with increased keeping qualities, and, it is believed, with a sharper definition of the chromatinic bodies.

ACKNOWLEDGMENT

The author wishes to thank Dr. C. F. Robinow for introducing him to and schooling him in basic bacterial cytological techniques. Gratitude is expressed also to Drs. Stuart Mudd and Harry E. Morton for helpful counsel during this investigation. For their extremely kind indulgence during the process of obtaining satisfactory photographs for the manuscript, I wish to thank Dr. E. D. DeLamater, Mr. Henry Stempen, and Mr. R. L. Chapman.

SUMMARY

A simplified cytological technique for demonstrating the chromatinic bodies within bacterial cells is presented and has been found to be generally applicable.

The technique consists of fixation in osmium tetraoxide vapor, treatment in hydrochloric acid, mordanting in dilute formaldehyde, and staining in aqueous basic fuchsin.

The technique is felt to possess advantages in terms of selectiveness, keeping qualities, and rapidity of preparation.

This technique is modified from the procedures of Robinow and DeLamater.

REFERENCES

BADIAN, J. 1933 Eine cytologische Untersuchung über das Chromatin und den Entwicklungszyklus den Bakterien. Arch. Mikrobiol., **4**, 409–418.

BISSET, K. A. 1948*a* Nuclear reorganization in non-sporing bacteria. J. Hyg., **46**, 173–175.

BISSET, K. A. 1948*b* The cytology of the gram-positive cocci. J. Gen. Microbiol., **2**, 126–130.

BISSET, K. A. 1948*c* Observations upon the bacterial nucleus. J. Hyg., **46**, 264–266.

BISSET, K. A. 1949 The nuclear cycle in bacteria. J. Hyg., **47**, 182–187.

BOIVIN, A. 1947 Directed mutation in colon bacilli, by an inducing principle of desoxyribonucleic nature: its meaning for the general biochemistry of heredity. Cold Spring Harbor Symposia Quant. Biol., **12**, 7–17.

CONN, H. J. 1946 Biological stains. Biotech Publications, Geneva, N. Y.

DELAMATER, E. D. 1948 Basic fuchsin as a nuclear stain. Stain Tech., **23**, 161–176.

DELAMATER, E. D., MESCON, H., AND BARGER, J. D. 1950 The chemistry of the Feulgen and related histo- and cytochemical methods. J. Investigative Dermatol., **14**. *In press.*

FLEWETT, T. H. 1948 Nuclear changes in *Bacillus anthracis* and their relation to variants. J. Gen. Microbiol., **2**, 325–333.

HILLIER, J., MUDD, S., AND SMITH, A. G. 1949 Internal structure and nuclei in cells of *Escherichia coli* as shown by improved electron microscopic techniques. J. Bact., **57**, 319–338.

JOHNSON, F. H., AND GRAY, D. H. 1949 Nuclei and large bodies of luminous bacteria in relation to salt concentration, osmotic pressure, temperature, and urethane. J. Bact., **58**, 675–688.

KLIENEBERGER-NOBEL, E. 1947 Morphological appearances of various stages in *B. proteus* and *coli*. J. Hyg., **45**, 410–412.

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

KNAYSI, G. 1944 The elements of bacterial cytology. Comstock Publishing Co., Inc., Ithaca, N. Y.

LEE, B. 1937 The microtomist's vade-mecum, ed. by J. B. Gatenby and T. S. Painter. 10th ed. P. Blakiston's Son and Co., Philadelphia, Pa. *Refer to* p. 28, 313, 316.

MCCLUNG, C. E. 1937 Microscopical technique. 2d ed. P. B. Hoeber, Inc., New York. *Refer to* p. 276, 559.

MORTON, H. E., AND ENGLEY, F. B., JR. 1945 The protective action of dysentery bacteriophage in experimental infections in mice. J. Bact., **49**, 245–255.

MORTON, H. E., AND PULASKI, E. J. 1938 The preservation of bacterial cultures. I. J. Bact., **35**, 163–183.

MUDD, S., AND SMITH, A. G. 1950 Electron and light microscopic studies of bacterial nuclei. I. Adaptation of cytological processing to electron microscopy; bacterial nuclei as vesicular structures. J. Bact., **59**, 561–573.

ROBINOW, C. F. 1942 A study of the nuclear apparatus of bacteria. Proc. Roy. Soc. (London), B, **130**, 299–324.

ROBINOW, C. F. 1944 Cytological observations on *Bact. coli*, *Proteus vulgaris* and various aerobic spore-forming bacteria with special reference to the nuclear structures. J. Hyg., **43**, 413–423.

ROBINOW, C. F. 1945 Nuclear apparatus and cell structure of rod-shaped bacteria. *Addendum to* R. J. Dubos. The bacterial cell. Harvard University Press, Cambridge, Mass.

SMILES, J., WELCH, F. V., AND ELFORD, W. J. 1948 The influence of antibacterial substances on the interaction of bacteria and bacteriophages. II. Optical studies of the penicillin effect. J. Gen. Microbiol., **2**, 220–227.

TULASNE, R., AND VENDRELY, R. 1947*a* Mise en évidence des noyaux bactériennes par la ribonucléase. Compt. rend. soc. biol., **141**, 674–676.

TULASNE, R., AND VENDRELY, R. 1947*b* Demonstration of bacterial nuclei with ribonuclease. Nature, **160**, 225–226.

TULASNE, R., AND VENDRELY, R. 1948 Cytology of staphylococci before and after treatment with penicillin. Nature, **161**, 316–317.