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Symposium on the Fine Structure and Replication of Bacteria and Their Parts

I. Fine Structure and Replication of Bacterial Nucleoids[1]

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INTRODUCTION

The genetic material in bacterial cells forms structures which are called *nucleoids* (27). Although from the very beginning there was no doubt that these structures are nuclei with respect to their function (28), their simple architecture and morphological appearance, which distinguish them from the type of nucleus present in higher organisms and which are also reflected by the classification of bacteria as protocaryotic rather than eucaryotic organisms (45), make a special term desirable. [The Greek prefixes "proto-" (from πρῶτος) or "arche-" serve better the idea of primitive or archaic as a contrary to "eu-" (meaning "well developed") than does the prefix "pro-" (from πρό) which has a more restricted meaning ("preceding with respect to time").] Nevertheless, terms such as "bacterial nucleus" are often used and may be accepted if there is no doubt regarding the writer's intention. The term *DNA-plasm* [not nucleoplasm (17)], meaning "region of the cell characterized by the presence of deoxyribonucleic acid (DNA)," is largely synonymous with "nucleoid" if applied to nuclear structures of intact bacterial cells, but in a broader sense can also be used for fragments of nucleoids, phage pools, DNA-containing regions of higher organisms, and even model systems.

Since a nucleoid is a single and, irrespective of shape, coherent structure, this term cannot be generally applied to the nuclear structures of the related Cyanophyceae. The cells of smaller cyanophyceae contain a single chromatinic region each. This region may be called a nucleoid, and probably contains one minimal genetic outfit in the form of a coherent DNA-containing structure as bacterial nucleoids usually do (see below). In the cells of larger cyanophyceae, however, several separate entities exist, representing a complete genome each, and acting together as a *chromatic apparatus* (2, 9, 43). All types of nuclear equivalents found in bacteria and cyanophyceae may be covered by the term *protocaryon*. (For discussion, *see also* 44, 45.)

At present, research on structure and replication of nucleoids follows several more or less independent lines. There are physiological and genetic approaches as well as the study of carefully isolated bacterial DNA. We are concerned here with evidence obtained from the most direct approach, that of cytological investigation.

Since 1956, when Robinow's review on light microscopic cytology of bacterial nucleoids (33) appeared, it has become clear that more information would be obtained mainly by electron microscopy and ultrathin sectioning. Since that

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time, numerous papers on ultrastructure of nucleoids have been published, and a critical survey on this literature has been presented recently by Brieger (3). His review gives the impression that two types of approach could help in overcoming the difficulties in understanding nucleoid structure, namely, integration of molecular with cytological data, and improvement of criteria for interpretation of ultrastructure; this report is devoted primarily to these types of approach. Finally, an attempt will be made to present a consolidated picture of nucleoid structure.

MOLECULAR STATE OF BACTERIAL DNA AS EVIDENT FROM ISOLATION EXPERIMENTS

If we briefly consider available evidence on the molecular state of bacterial DNA as obtained from isolation experiments, we must first mention the work of Kleinschmidt and co-workers (20, 21, and earlier publications). They succeeded in spreading the DNA from bacterial cells and protoplasts at an air-water interface. Electron micrographs show that the DNA of these bacteria apparently forms one or very few molecular strands. There are no free ends nor kinks nor other irregularities in shape. Cairns (4, 5), in his autoradiographic studies of labeled and carefully isolated *Escherichia coli* DNA, found molecules which were 1,100 to 1,400 μ long and sometimes appeared as closed circles. Beyond that, he found structures which apparently are replicating ring forms. By pulse-labeling, it could be confirmed that in rapidly growing bacteria replication of DNA goes on continuously (which means that one cycle follows another practically without delay) and proceeds along the DNA unit with constant velocity. This DNA unit is usually called the *bacterial chromosome*, but according to Ris (32) it should be called a *genophore* to avoid confusion with the term chromosome as applied to complex structures within the nuclei of eucaryotic organisms.

Replication of this unit starts at one point only, and this point can be characterized with respect to its position on the genetic chart (26). The unit apparently consists of a two-stranded helical DNA molecule (4, 5). Its manner of replication is semiconservative and involves separation of the polynucleotide strands (25).

Further conclusions can be drawn if we combine the following well-established data and observations, but it should be mentioned that some of them may be valid for *E. coli* only.

E. coli cells contain about 1.2×10^{-14} g of DNA during rapid exponential growth; in the stationary phase they contain about 0.7×10^{-14} g (15). At high growth rates, the cells contain between two and four nuclei; at low rates, between one and two (27, 40). In a growing population, 10 to 30% of the cells have completed nucleoid division but have not yet completed cell division. At high growth rates, DNA replication goes on continuously (24, 39, 47*a*), and within the individual cycle of an individual cell proceeds linearly with time (4). The age distribution of cells in a growing population is as described mathematically by Powell (29). The minimal length of a DNA unit is 1,100 μ (5).

If we compute these data, we come to several conclusions. (i) There is only one DNA unit, resting or replicating, per nucleoid. (ii) The DNA unit consists of not more than two polynucleotide strands. (iii) Only 20% or less of a generation time is available for nucleoid division after completion of the replication cycle of the DNA unit. This means that either the separation of daughter nucleoids takes place rapidly and immediately after the DNA unit has finished its replication cycle or that replication of the DNA and nucleoid division proceed almost simultaneously. Cytological evidence supports the latter possibility. If we would make the assumption that replicated regions of the DNA unit remain in close contact side by side as apparently four-stranded DNA, this type of DNA could not make up more than 10 to 20% of the total DNA. (iv) The maximal length of the DNA unit compatible with these premises is 1,400 μ. The conclusions on two-strandedness and maximal length are in accordance with observations by Cairns (4, 5) reported above.

If DNA replication and nucleoid division take place simultaneously, the molecular diagram (Fig. 1) of the replication of the bacterial chromosome established by Cairns (5) should have its cytological counterpart in a nucleoid being almost permanently in a state of division.

STATE OF INTRACELLULAR BACTERIAL DNA AS REVEALED BY EXPERIMENTAL ELECTRON MICROSCOPY, AND PRESERVATION OF NUCLEOID STRUCTURE DURING PREPARATION OF CELLS FOR SECTIONING

Next, we shall consider the fine structure of the nucleoid as seen in ultrathin sections, because sectioning, especially serial sectioning, is the most promising way to reveal the arrangement of the bacterial chromosome inside the cell. It should be kept in mind, however, that interpretation of ultrastructure is as reliable as are the criteria on which it is based, and various criteria which may have their merits in light microscopy have to be looked at with reservation or are completely worthless in the macromolecu-

lar range. Even conclusions from the behavior of single molecular species in in vitro reactions or the behavior of incomplete model systems have to be drawn with considerable caution, and usually cannot replace the approach by experimental and quantitative electron microscopy. The latter (at least in some instances) is able to provide independent criteria for interpretation, and eventually also data on the physical and chemical state of cellular constituents which are not easily obtainable by a biochemical approach. In any case, criteria for interpretation have to be developed separately for every type of compound structure.

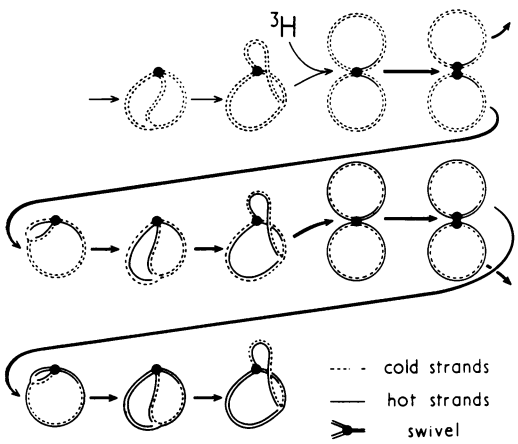


FIG. 1. *Diagrammatic representation of the replication of a circular bacterial chromosome (from Escherichia coli K-12, Hfr thy$^-$). Each chromosome is shown as a circular double line representing a two-stranded DNA molecule. Replication proceeds in a semiconservative manner. DNA synthesis is indicated as growth of two "hot strands" after addition of tritiated thymidine to the growth medium. To allow for uncoiling of the parental Watson and Crick helix during replication, existence of a "swivel" is postulated. At this structure, the unduplicated part of the chromosome is assumed to rotate with respect to the rest. From Cairns (5).*

Bacterial cells which are prepared for ultrathin sectioning undergo various treatments that in many instances cause considerable distortion and disorganization of nucleoid structure, and I shall try to summarize available evidence concerning this point.

In the usual sequence of events when bacteria are prepared for ultrathin sectioning, the cells are fixed in a solution of osmium tetroxide in a suitable buffer system. Next follows treatment with an aqueous solution of uranyl acetate. The cells are transferred immediately into an acetone-water mixture, dehydrated in pure acetone, and embedded in plastic.

Osmium Fixation

The first step, osmium fixation, kills the cells and stabilizes the cytoplasm. Osmium tetroxide does not react with DNA (1), nor are DNA molecules affected in other ways by this agent (12). Within bacterial cells, the DNA-containing structures apparently are not fixed by osmium tetroxide, because this type of fixation does not prevent subsequent gross coagulation of DNA material. Observations with the phase-contrast microscope show that there is apparently no osmium deposit in the nucleoid region (Fuhs, *unpublished data*), and this may be taken as an argument against the possibility that bacterial DNA is associated with histonelike protein, since histones combine with osmium tetroxide (1). Further arguments concerning this point are presented later in this review.

Osmium fixation of bacterial nucleoids was studied in detail by Ryter and Kellenberger (38). Their work included development of a refined technique, which is called the RK technique after both authors. It consists of osmium fixation in the presence of amino acids and calcium ions. If RK fixation is performed properly, the structures obtained are strikingly different from those obtained by ordinary fixation. After ordinary fixation there are numerous small aggregations of DNA fibers (Fig. 2 and 3), whereas after RK fixation the nucleoid shows a nearly homogeneous appearance at low and moderate resolutions (Fig. 4 and 5).

The RK technique is somewhat critical, and at least in the case of *Bacillus subtilis* and *E. coli* the instructions given by the authors have to be followed exactly. Several workers claim to have used RK fixation, but due to minor modifications obtained results (resembling Fig. 2) which indicate that RK conditions were not obtained. The same apparently holds for the suspected difference in response of gram-positive and gram-negative bacteria, at least as far as *B. subtilis* and *E. coli* are concerned. On the other hand, RK conditions may be different for different species and physiological states of bacteria, and thus may need redefinition in certain cases.

The amino acids responsible for the RK reaction are those which react with osmium tetroxide, and changes in mass density, revealed by phase contrast microscopy, indicate that osmium is introduced into the nucleoid under RK conditions (Fuhs, *unpublished data*). It has been found that a calcium-containing mixture of amino acids, osmium tetroxide, and high-molecular-weight DNA is able to form a gel-like precipitate in

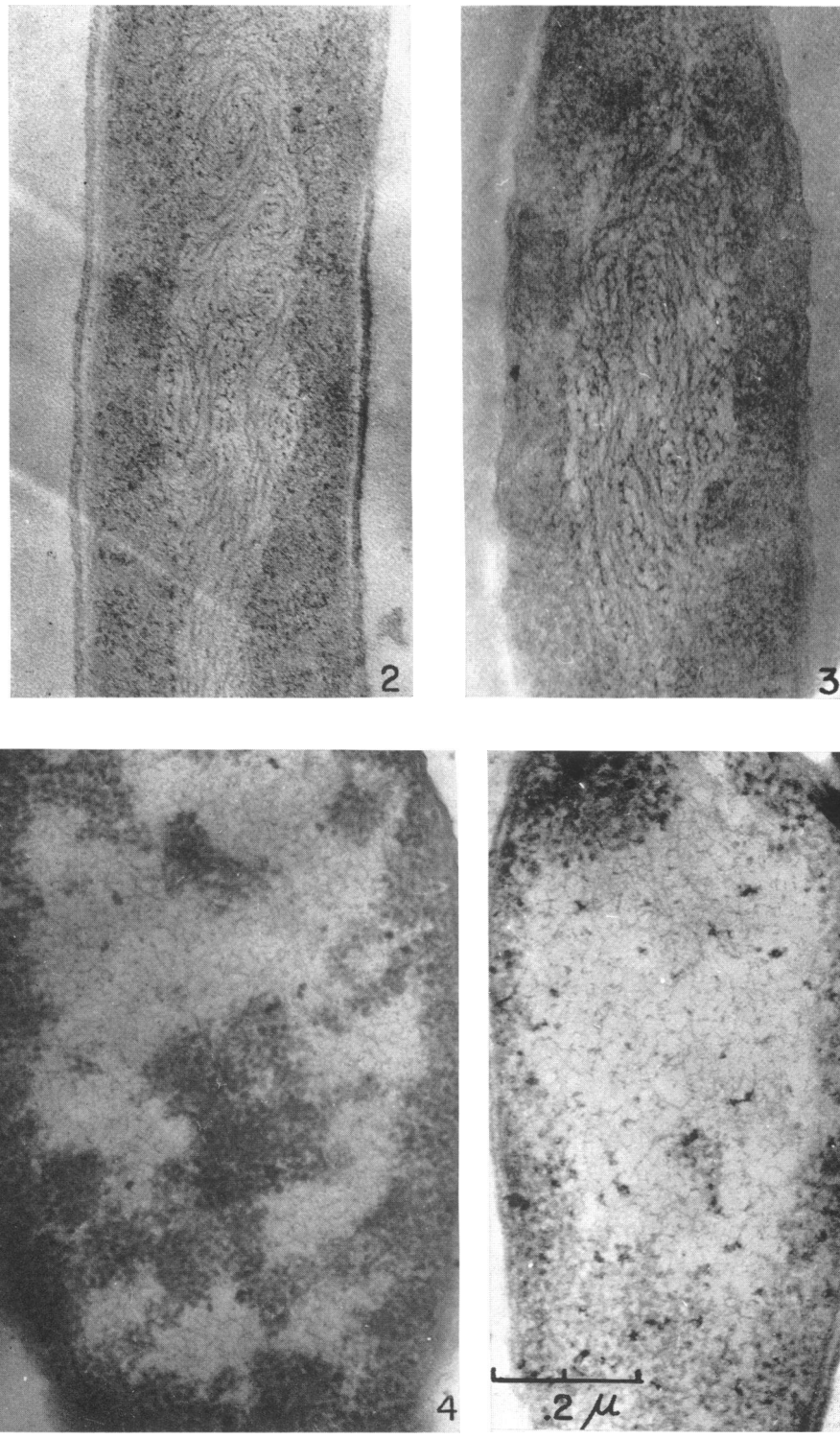


FIG. 2 and 3. *Bacillus subtilis (Fig. 2) and Escherichia coli (Fig. 3), embedded and sectioned after ordinary osmic or osmic-chromic fixation and uranyl treatment. The nucleoids contain numerous fibers which are formed by random aggregation of parallel-oriented DNA helices.*

FIG. 4 and 5. *Bacillus subtilis (Fig. 4) and Escherichia coli (Fig. 5) after RK fixation and uranyl treatment. The nucleoids show a more homogeneous appearance. Black deposits, which also appear outside the cells, are artifacts resulting from an additional uranyl treatment after sectioning.*

which the DNA molecules are prevented from aggregation upon addition of uranyl ions and upon dehydration (41, 42). DNA itself seems not to be chemically involved in the reaction, because only two of the amino acids (arginine and lysine) are able to combine with DNA, and even their affinity to DNA phosphate groups is too low to compete effectively with the ligands already present or with the calcium ions present in the reaction mixture.

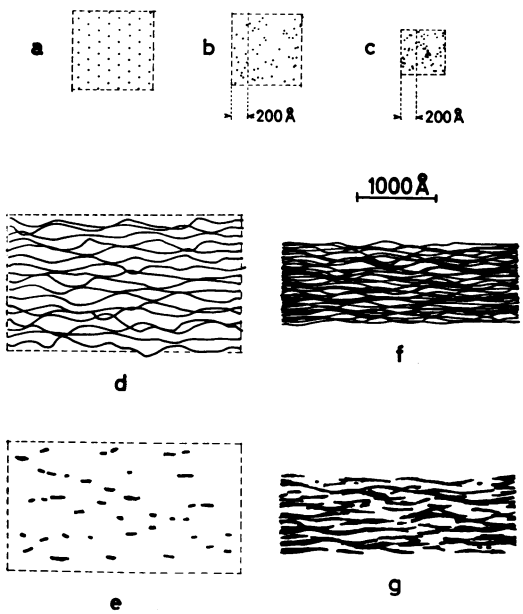


FIG. 6. *(a) Diagrammatic representation of a pattern of DNA helices (helices cross-sectioned) in a nucleoid. Spacing of helices is about the same as in the living state. (b) The same pattern after RK fixation, dehydration, and embedding; (c) the same after ordinary osmic-chromic fixation, dehydration, and embedding; (d and f) longitudinal sections of 200 A thickness of the patterns b and c, respectively; (e and g) approximate representation of the optical artifacts resulting from patterns d and f, respectively, at 30 A resolution. (e) Resembles the pattern of Fig. 4 and 5; (g) that of Fig. 2 and 3. From Fuhs (11).*

The most striking effect of RK fixation is that during subsequent dehydration the cells retain almost their original size, their diameter being reduced by not more than 10% compared with about 40 to 50% after ordinary fixation. Certainly, this has considerable influence on apparent nucleoid structure (11). What is expected to occur is shown in Fig. 6. Figure 6a represents a section of a nucleoid with cross-sectioned DNA helices being oriented parallel to each other at about equal distances. The relation of helix diameter to distance between helices in this figure is the same as in the living state. Upon reduction of surface charge due to uranyl complex formation their arrangement becomes random, and upon dehydration the diameter of the bundle decreases by 10% after RK fixation (Fig. 6b) and by 46% after ordinary osmium fixation (Fig. 6c). Upon heavy shrinking (as in Fig. 6c) there appears a marked tendency for random aggregation, which is favored also by uranyl action. If we cut both bundles into longitudinal sections of the same thickness (e.g., 200 A, as demonstrated in Fig. 6b and 6c), the section of the RK-fixed cell (Fig. 6d) will contain fewer helices than the other (Fig. 6f); and, if electron-microscopic resolution is not sufficient to resolve individual helices clearly, as is usually the case, optical artifacts will result (Fig. 6e and 6g). This offers a quite reasonable explanation for the different patterns obtained after RK and normal fixation techniques. Certainly, RK fixation gives results which are closest to the living state, but a disadvantage is that the direction of the DNA fibers is usually difficult to recognize, whereas their orientation becomes very obvious if side-by-side aggregation of fibers, to a certain extent, is allowed to occur.

It has been reasoned that the aggregates in reality are preformed units which are destroyed by RK fixation, but this can be shown to be incorrect by investigation of serial sections. In these sections the aggregates possess quite different diameters, and the pattern changes from one section to another, if cross sections are observed (Fig. 7).

Action of Uranyl Ions

After fixation, bacteria are treated with aqueous solutions of uranyl acetate, and this leads to the formation of a complex between uranyl ions and intracellular nucleic acids (48). The use of aqueous solutions is essential for specificity of the reaction as well as for extensive complex formation which is necessary to prevent subsequent damage by dehydration, as pointed out below (*see also* 11). The DNA molecules and other cellular constituents containing nucleic acids are stained and become visible in the electron microscope.

In vitro studies on complex formation between DNA and uranyl ions revealed a high initial association constant (range of 10^6), which rapidly decreases with increasing saturation of the polymer (14, 48). This type of ion interaction indicates that uranyl ions are bound in a saltlike manner to the phosphate groups of DNA.

Ligands which are present at these sites of intracellular DNA in vivo are expected to be replaced by uranyl groups upon treatment of

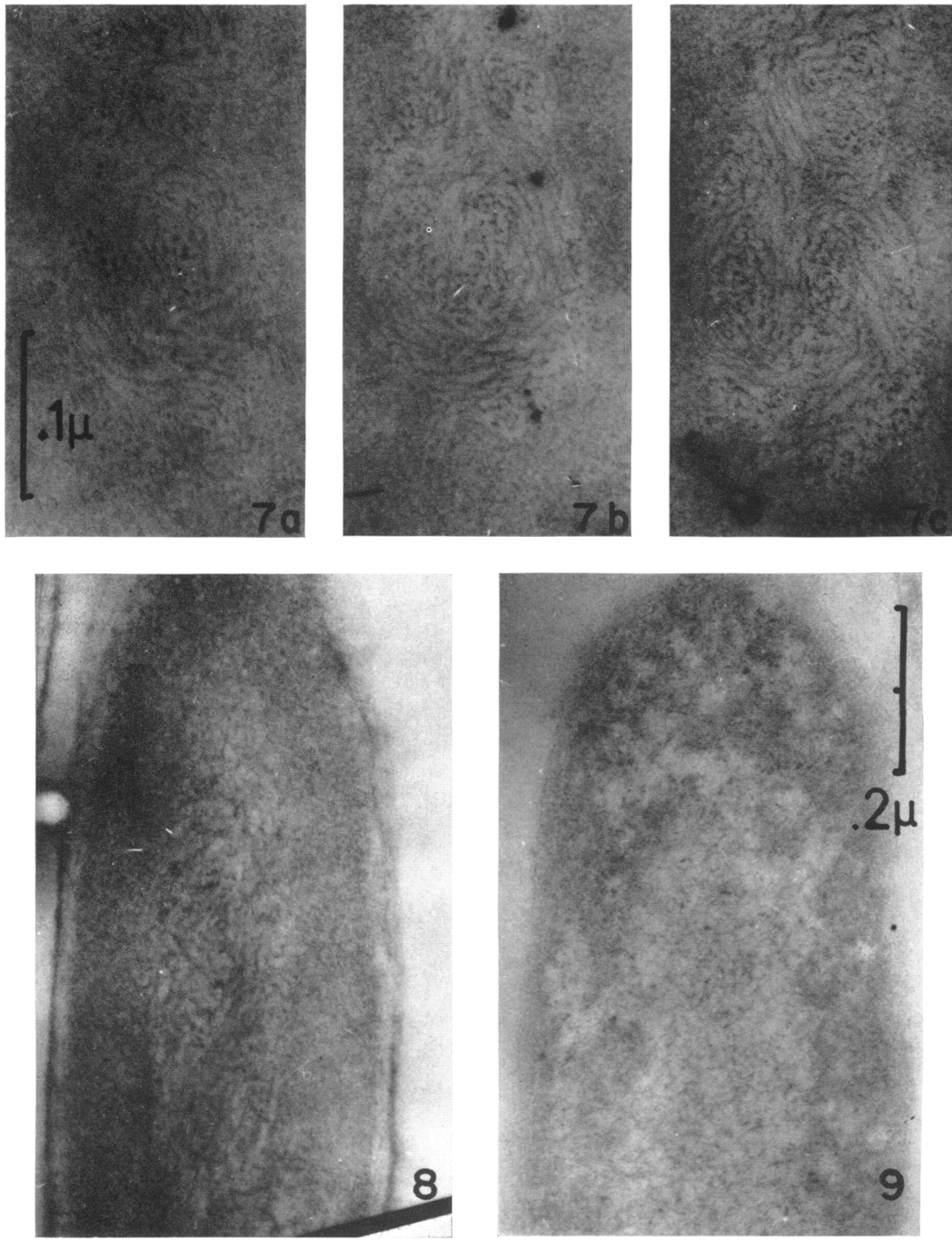
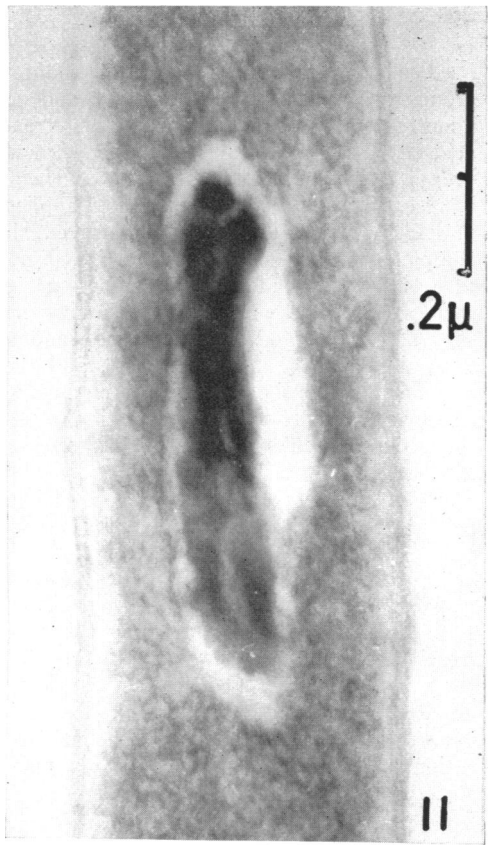


FIG. 7. *Part of a nucleoid of Bacillus subtilis, three consecutive serial sections. A bundle of DNA fibers (aggregates) penetrating three planes of section. The aggregates possess different diameters, and only in very few cases can an individual aggregate be identified in two adjacent sections (section thickness, 200 A).*

FIG. 8 and 9. *Bacillus subtilis (Fig. 8) and Escherichia coli (Fig. 9), denatured state of DNA; compare with native state as shown in Fig. 2 and 3.*



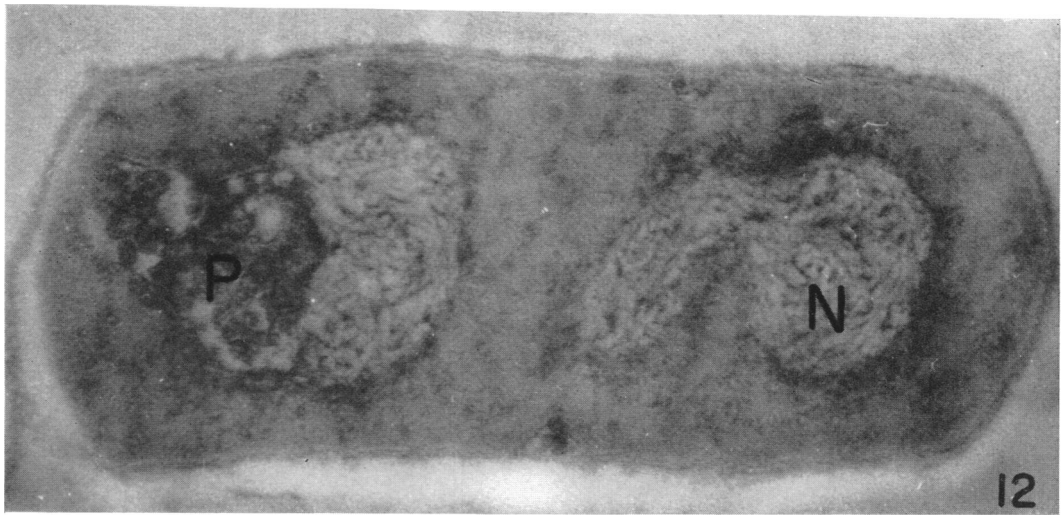


FIG. 10. *Bacillus subtilis after osmic-chromic fixation, no uranyl treatment. Artificially condensed state of DNA in the nucleoid.*

FIG. 11. *Bacillus subtilis, after fixation partly dehydrated in 70% ethyl alcohol, then treated with 1.5% uranyl nitrate, dissolved in 70% ethyl alcohol, further dehydrated in acetone, embedded in Vestopal. Intermediate state of artificial condensation of DNA material. The solid strands in this preparation are equivalent to the bundles described in the text.*

FIG. 12. *Bacillus subtilis during rapid exponential growth (30-min generation time). Nuclear and ribosomal pattern obscured by nonribosomal RNA, which also forms a halo around the nucleoid (N). Adjacent to the nucleoid is a plasmalemmosome (P). Compare with Fig. 2 (slowly growing cell).*

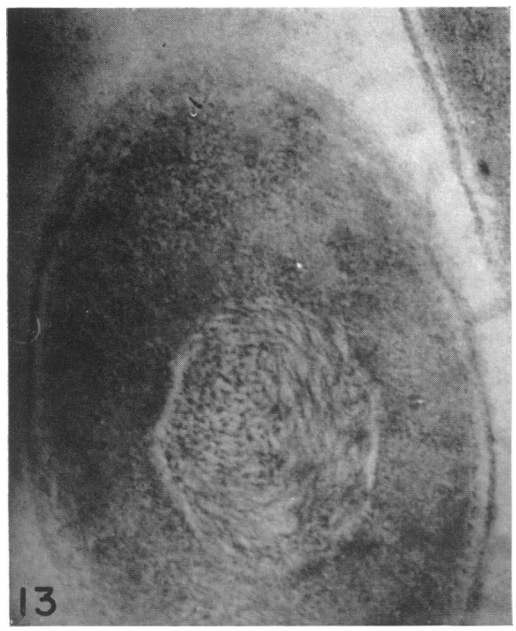
fixed cells with uranyl solutions. Indeed, if uranyl groups are introduced in this way and subsequently removed by ethylenediaminetetraacetic acid (EDTA), the affinity of the DNA towards uranyl is increased about 40-fold, and a slight increase of affinity is observed after application of EDTA alone. This effect is reversed almost completely by subsequent application of a mixture of calcium and magnesium ions and polyamines (14). These results indicate that metals such as calcium and magnesium are among the natural ligands of bacterial DNA, and the remainder may consist of organic compounds such as polyamines. There is no necessity to assume that high-molecular-weight ligands are associated with bacterial DNA.

Several authors presume that uranyl ions affect the physical state of DNA by decreasing stability of helical conformation or by cleaving phosphate diester bonds, in this way eventually causing disorganization of nucleoid structure. Experimental evidence concerning this point is clearly against this assumption (12). At the same time, it was found that intracellular DNA is in a state in which it is effectively stabilized against denaturation (i.e., against loss of helical configuration due to separation of polynucleotide strands).

Conditions for Denaturation of Intracellular Bacterial DNA

The electron micrographs show how denaturation of DNA becomes apparent in ultrathin sections (Fig. 8 and 9). The DNA aggregates in orderly arrangement, as seen in the control preparations (Fig. 2 and 3), have completely disappeared and are replaced by a chaotic pattern formed by randomly coiled single-stranded DNA.

Denaturation of dissolved DNA in vitro occurs almost immediately after the temperature has been raised above a certain point (dependent on base composition of the DNA). Denaturation can also be achieved by high or low *p*H or by replacing water as solvent, e.g., by formamide. Such treatment, however, is not sufficient for denaturation of intracellular DNA of bacteria, unless the DNA has been degraded to a certain extent beforehand, e.g., by prolonged heating of the fixed cells to a temperature slightly below the melting point of their DNA (12). It can be calculated that about 1,000 chain breaks must be introduced into the bacterial chromosome for denaturation to take place. On the average, this corresponds to one chain break in each of the DNA fibers which are oriented in the nucleoid more or less in its longitudinal axis. The assump-



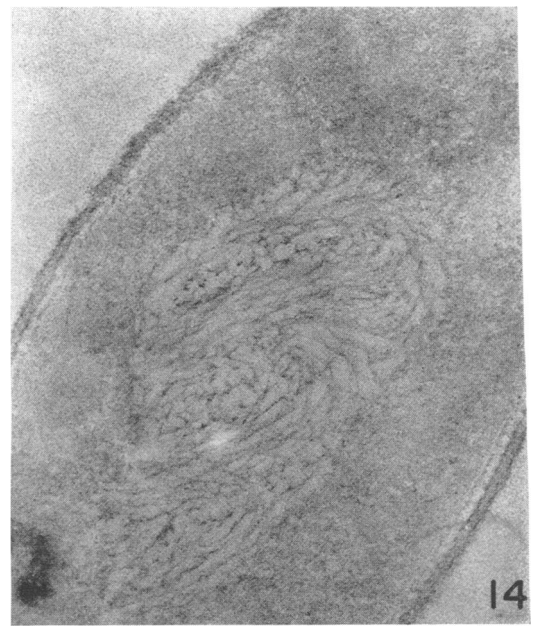


FIG. 13. *Bacillus subtilis, almost cross-sectioned cell.*

FIG. 14. *Bacillus subtilis, featherlike structures. This pattern is found often in obliquely sectioned cells. From Fuhs (13).*

tion provides a reasonable explanation of the behavior observed, if we assume that all these fibers form a single continuous DNA molecule in living bacteria.

The stabilization by molecular size and shape of intracellular bacterial DNA explains also why the DNA of thermophilic bacteria does not need a special base composition to be protected against thermal denaturation (22). Protection by shape is much more effective. But, on the other hand, thermally induced chain breaks which occur readily below melting temperature of DNA (6, 8), and the functioning of repair mechanisms, may be of importance for the survival of these bacteria at elevated temperatures.

As far as conservation of nucleoid structure is concerned, these experiments (12) show that neither osmium tetroxide fixation (including RK technique) nor uranyl treatment affects the integrity of the bacterial DNA unit. This means that the number of possibilities for producing gross alterations in nucleoid structure by use of these techniques is reduced to aggregation phenomena as they are discussed in other chapters of this review, and that higher degrees of order, if found in nucleoids, have to be regarded as real structures rather than as artifacts.

Absence of Condensed States of DNA in Bacteria

Having dealt with the chemical and physical states of intracellular bacterial DNA, according to the classification chosen by Kellenberger (17), I shall consider its organizational state.

A question which has been discussed for several years concerns the possibility that there exist condensed states of DNA in the bacterial cell. The discussion emerged from the fact that, before aqueous solutions of uranyl salts were applied, the sectioned nucleoids appeared in a condensed form (Fig. 10). That these condensed states are artifacts caused by dehydration could be shown in three different ways. Schreil (41, 42) found a striking similarity in behavior of artificial DNA plasms and of bacterial nucleoids. Since chemical states of free and intracellular DNA are somewhat different, however, more convincing evidence is the observation that, upon partial dehydration and subsequent application of uranyl solutions of high ionic activity, intermediate states of condensation appear (11). The only explanation possible is that DNA material undergoes gross coagulation unless uranyl groups have been introduced, and that upon partial dehydration and subsequent application of uranyl ions this process stops at an intermediate stage (Fig. 11). Quantitative analysis shows that coagulation is prevented if about 50% or more of the phosphate groups of DNA are neutralized by uranyl groups (14). The third type of evidence is that condensed states of DNA present in phage heads are preserved during preparation, whereas the DNA of the intrabacterial phage pool appears in its native hydrated state (19). If we consider all experimental evidence and electron micrographs obtained from different bacteria in different physiological and developmental states, as reported by numerous authors, we can safely state that condensed states of DNA are nonexistent in normally developing bacteria. (For detailed discussion, *see also* 17 and 18.)

General Characteristics of Nucleoid Fine Structure

With all this in mind, we are able to describe the fine structure of the bacterial nucleoid in greater detail. The interpretation is to a large extent based on about 100 series of sections of *B. subtilis* and *E. coli* nucleoids, complete series consisting of 8 to 12 sections.

In the sections, the nucleoid appears simply as a region of the cell which is characterized by the presence of DNA and the apparent absence of other macromolecular components such as ribosomes (Fig. 18). However, nonribosomal RNA appears in rapidly growing cells as a structureless background in both nuclear and cytoplasmic regions, partially obscures nucleoid structure and even the ribosomal pattern, and may form a dense halo around the nucleoid (Fig. 12).

The most characteristic and most general feature of nucleoid structure is a parallel arrangement of DNA fibers or aggregates. They form bundles which must contain up to 500 molecular helices of DNA. They are oriented preferably in the long axis of the bacterium (Fig. 2 and 3). Cross-sectioned cells (Fig. 13) usually show cross-sectioned bundles surrounded by obliquely sectioned aggregates. Sometimes this pattern (Fig. 14) is repeated several times, especially in germinating spores (featherlike structures; *see* 35). That this type of pattern cannot be regarded as part of a supercoiled system could be shown by model experiments and serial sectioning (13). Series of longitudinal sections show bundles crossing each other in different planes (Fig. 15). A more detailed analysis reveals that there are two bundles wrapped around each other, and this pattern could be detected in several nucleoids; but in all these nucleoids there are also regions which show a more complicated architecture and which are difficult to analyze.

Sometimes bundles turn backward at one end of a nucleoid (Fig. 16), but there are also points where one or two adjacent bundles seem to end freely (Fig. 17); sometimes there are turning points at both ends of a nucleoid. If turning

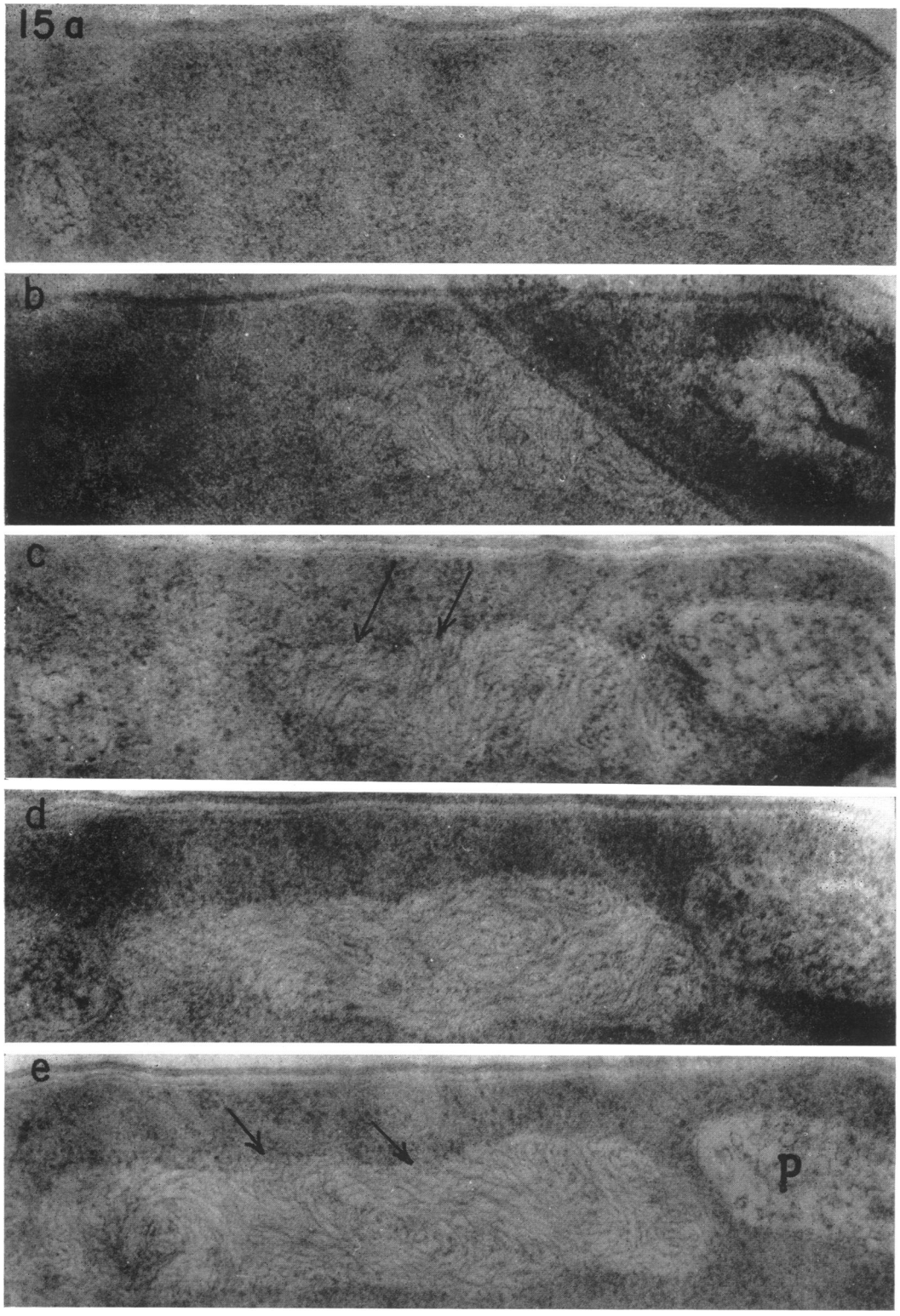


FIG. 15 (*a–e*) *Bacillus subtilis, serial sections. DNA fibers arranged in bundles crossing each other in different planes* (*arrows*). *P, plasmalemmosomes.*

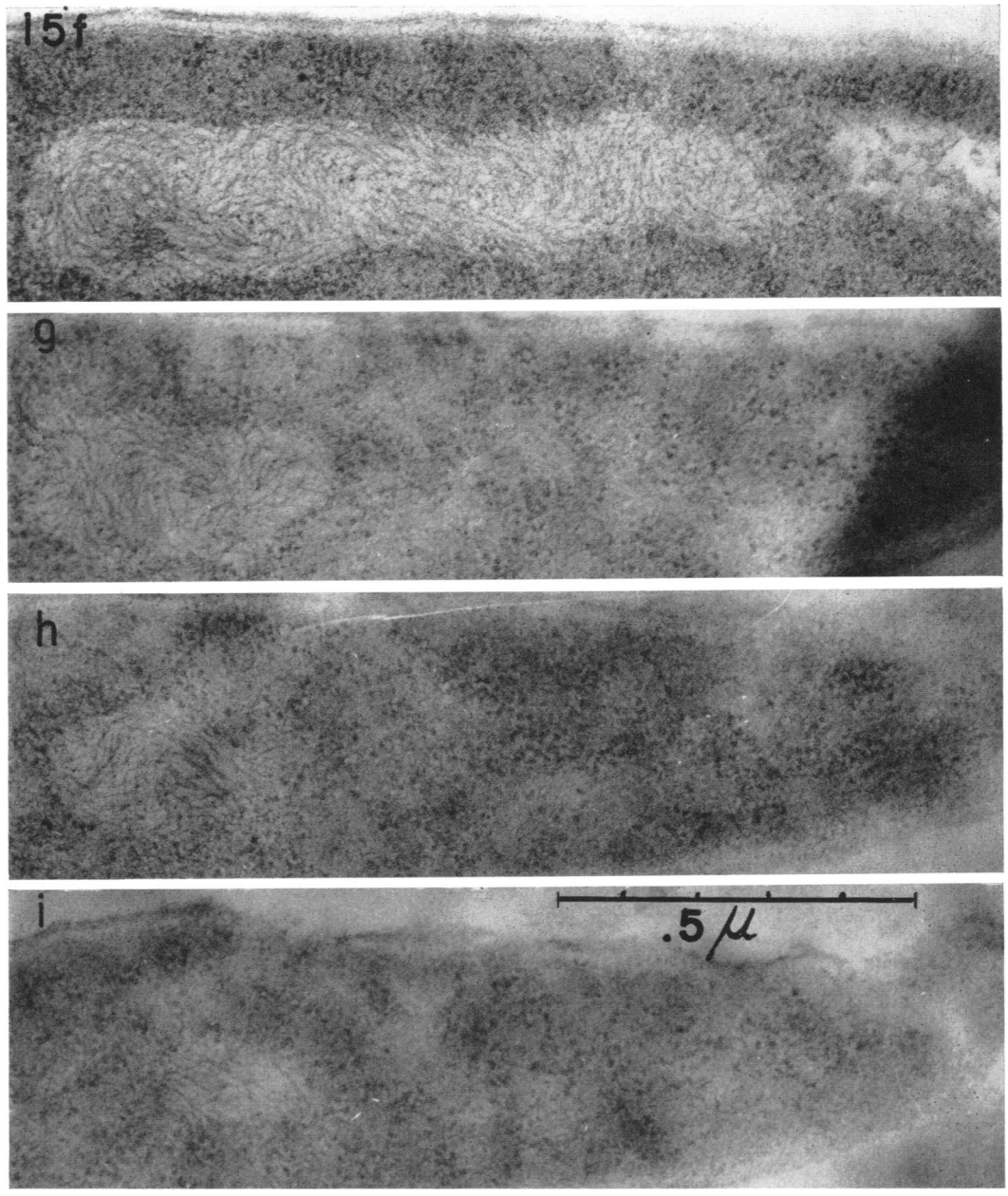


FIG. 15 (*f–i*) *Same series of Bacillus subtilis continued.*

points and free ends could be observed with equal frequency, one might tentatively assume that the basic structure in the nucleoid is a cyclic bundle or a multistranded ring, because turning points can be recognized only if the bundle happens to change its direction in a plane not very different from the plane of section. But this idea had to be abandoned after we had the opportunity to prepare serial sections of protoplasts of *B. subtilis* (prepared and kindly supplied by L. Caro, Institut de Biologie Moléculaire, Geneva, Switzerland). The nucleoids in these protoplasts consist of a bundle of parallel fibers and nothing else, and thus show the simplest pattern ever obtained (Fig. 18). No turning points were found, and this is clearly contrary to what we expect of a multistranded ring.

If we take this into account and confine our

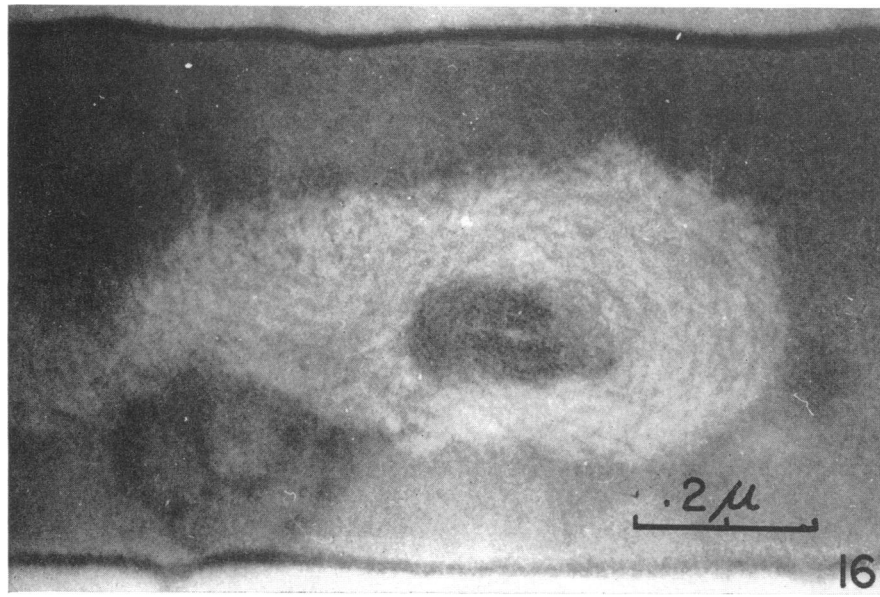


FIG. 16. *Bacillus subtilis, turning point of a bundle at right end of the nucleoid.*

choice to models which are easy to unfold and are able to replicate in accordance with the molecular model proposed by Cairns (Fig. 1), we have to assume as the most probable type of organization a structure which consists of a bundle of fibers folded back and forth (Fig. 19). This is what was proposed by Kellenberger in 1960 (16), the only major difference being that the presence of numerous non-DNA-linking groups at the ends of the structure could not be demonstrated thus far, and the existence of several types of linking groups can be excluded from evidence cited above. But, on the other hand, as long as we do not know whether the part of the bacterial chromosome which is to be replicated rotates in its entire length around its own axis, and if it does, how it succeeds in doing so, the postulate is still effective that there should exist linking groups or points of breakage and reunion which allow parts of the chromosome to rotate freely in order to uncoil the Watson and Crick helix more easily. A minor difference of the revised model, compared with the original, is that it is not twisted around its own axis. In single sections the impression of a single twisted bundle is often produced, but in such cases it is apparent from serial sections that two bundles are wrapped around each other.

The nuclear patterns observed in a large number of cells of *B. subtilis* and *E. coli*, resting and growing, are different from cell to cell, and in most cases are complicated and difficult to analyze (Fig. 20). However, the numerous patterns are obviously built according to the few simple rules mentioned, and there is no indication that other principles of organization exist which are basically different from those reported above.

From the experiments of Whitfield and Murray (47) and of Preusser (30, 31), it is evident that, following changes in the environment, the nucleoids change their shapes within a few minutes from a compact to a more tapelike appearance. Ryter (36) has already shown that these tapes are part of a single coherent nucleoid structure. There is no difficulty in understanding this change in appearance, because each of these tapes consists of a single bundle of DNA fibers (Fig. 21), and the nucleoids may change their shapes without the basic pattern being altered appreciably.

Certainly the nucleoid is a highly ordered structure, but this type of order must be described in terms of topology rather than geometry.

NUCLEOID REPLICATION

Most patterns observed represent phases of nucleoid division, and nucleoid division, as seen in the phase-contrast microscope (23, 34), seems to be accompanied by rapid changes in shape of nucleoids. Certain movements may be necessary for nucleoid division, and all these observations are compatible with the simple but very flexible pattern which has been observed.

A first attempt to analyze phases of nucleoid division revealed in one cell a system of three

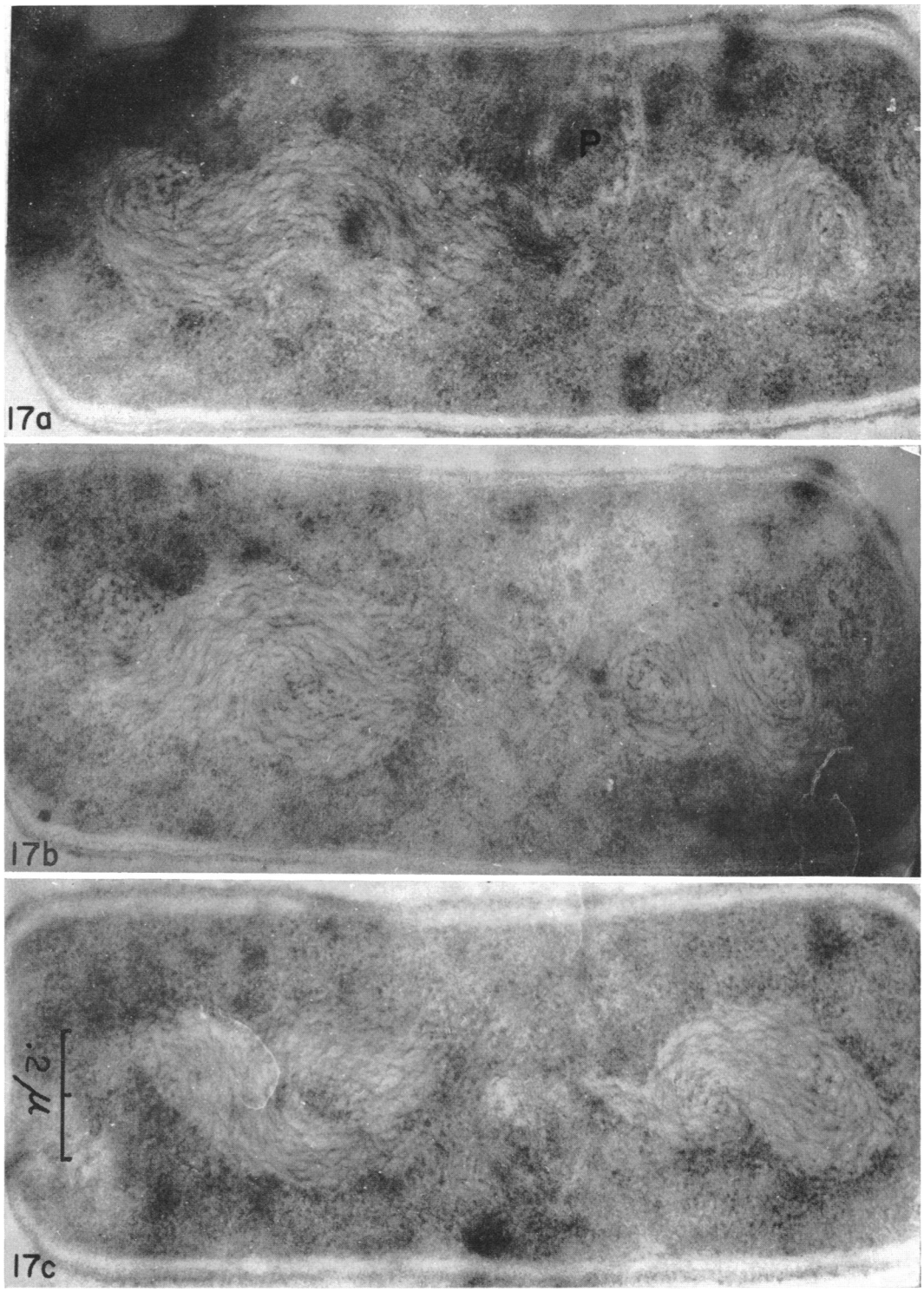


FIG. 17. *Bacillus subtilis, a short series of thicker sections. The nucleoid on the left side consists of a bundle which originates near the center of the cell (attachment to the plasmalemmosome?), proceeds to the left pole of the cell, turns backward, and seems to end near the center of the cell (free ending?). The nucleoid on the right side shows a similar arrangement. From Fuhs (10).*

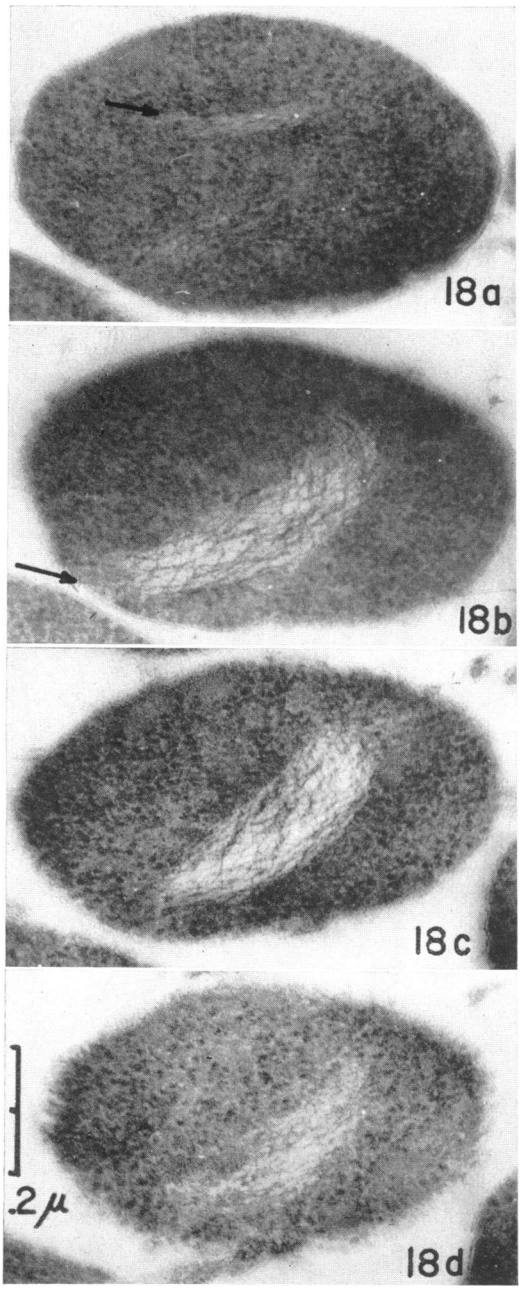


FIG. 18. *Bacillus subtilis, protoplast, serial sections, all sections containing nuclear structures are shown. The nucleoid forms a single bundle of fibers. One end of bundle apparently is attached to membrane; the other forms a narrow tail (arrows).*

circles touching each other in two points. In another case, two nucleoids of two sister cells looked quite different at first sight, but upon more detailed analysis revealed the same topological pattern consisting of two circular bundles and a third one with an open end. One may be tempted to compare this pattern with that of the replicating bacterial chromosome (Fig. 1), with one ring being replicated and two others formed; this conclusion would be premature, because in every nucleoid analyzed thus far there are regions where two bundles are oriented parallel to each other and come into close contact for a short distance. In such cases, they cannot be clearly distinguished from each other by the observer, and, as single DNA helices cannot be traced with our present techniques, it is by no means clear what happens in these regions. In any case, the variable and often complicated geometry of replication patterns may result from accidental variation of a general and topologically rather simple replication scheme.

NUCLEOIDS AND PLASMALEMMOSOMES

Finally, I should like to comment on a problem which has been raised recently. Apparently,

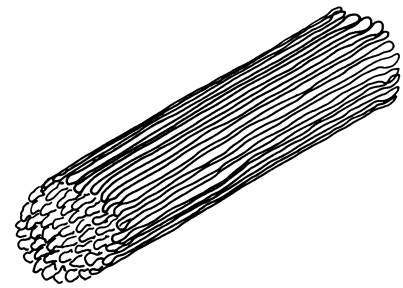


FIG. 19. *Semidiagrammatic representation of the model of nucleoid structure originally proposed by Kellenberger (16) and modified as described in the text. In reality there are 10 to 20 times as many molecular strands as indicated in the diagram. For topological interpretation and a possible mode of reduplication, see original publication (16).*

in many instances there is an intimate contact between the nucleoid and the cytoplasmic membrane, either directly, as in protoplasts, or mediated by plasmalemmosomes (7), which can be regarded as invaginations of the cytoplasmic membrane (mesosomes, lamellar bodies, membranous organelles; *see* 3 for references). As shown by van Iterson in 1960 (46), both nucleoids and plasmalemmosomes can come into close contact or even penetrate each other. Ryter and Jacob (37) succeeded in demonstrating a contact between nucleoids and plasmalemmosomes in each of more than 20 serially sectioned cells of *B. subtilis*. This observation could essentially be confirmed by careful analysis of 58 series from our laboratory. In only three cells and two protoplasts was there apparently no contact between nucleoid and membrane, but the possibility cannot be excluded that the connection is established by very few or even a single molecular

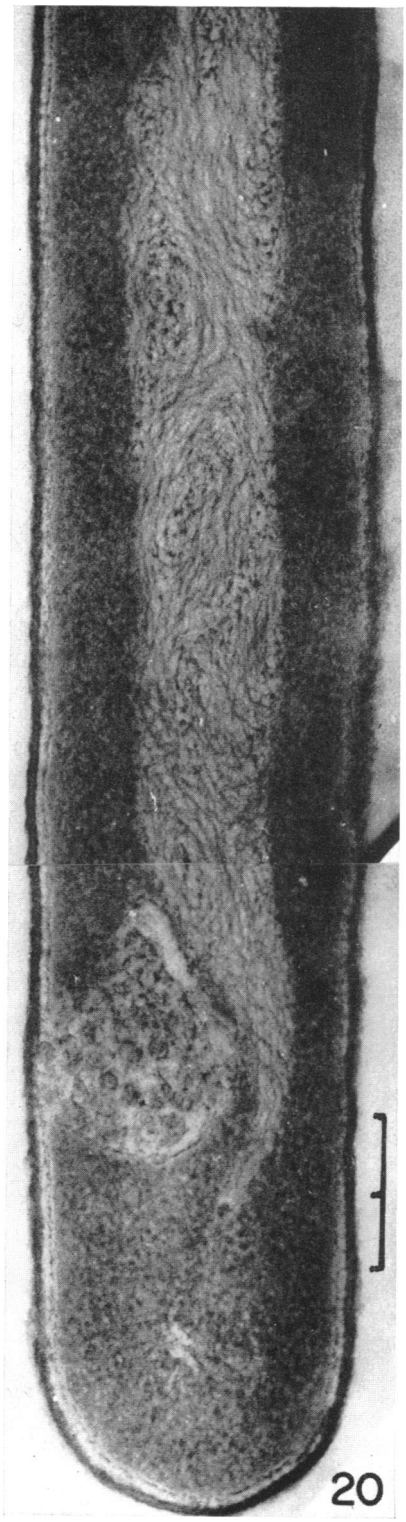


FIG. 20. *Bacillus subtilis; complicated internal structure of the nucleoid. This section is taken from a complete series.*

fiber of DNA which would inevitably escape detection. In two protoplasts, only an extremely delicate channel connected nucleoid and membrane, and it was impossible to decide whether there existed an intrusion of the membrane or an extrusion of the nucleoid.

I also tried to find out whether plasmalemmosomes are involved in the process of nucleoid division. But apparently there are considerable differences in the number of plasmalemmosomes per cell even in different strains of *B. subtilis*, and in my material there was no positive correlation between the size of a nucleoid and the number of plasmalemmosomes adjacent to it. Of 29 cells taken from exponential growth, only 3 smaller nucleoids were in contact with two plasmalemmosomes (one at each pole). Nucleoids with one polar plasmalemmosome only were predominant over the whole range of nucleoid and cell lengths, and the same holds for those

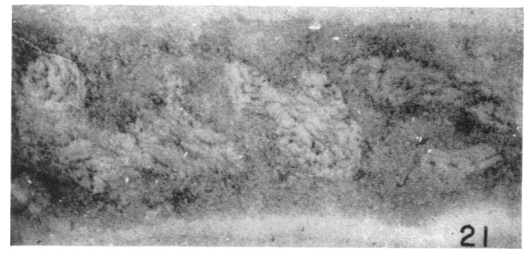


FIG. 21. *Bacillus subtilis, rapidly growing cell similar to that in Fig. 12, section taken from a complete series. The cell contains tapes of DNA plasm forming a complicated coherent structure (a nucleoid in a stage of replication). The tapes are identical with bundles of DNA fibers. From Fuhs (13).*

nucleoids the center of which is in contact with a plasmalemmosome (13).

Searching for special structures or structural regularities at the point of contact, I found that plasmalemmosomes are occasionally connected to the end of a bundle of DNA fibers, but there were also free ends of such bundles, and other plasmalemmosomes touching a DNA bundle in a different way.

These observations are not in favor of the view that plasmalemmosomes play an active part in nucleoid division.

CONCLUDING REMARKS

Ten years ago it was reasonable to assume that the easiest way of elucidating structure and replication of nucleoids would consist in application of refined microscopic techniques. Since then, however, most remarkable contributions to our understanding of the genetic structure in bacteria came from experiments quite different

in design, and cytological investigation has just reached a point where gross misinterpretation of structures observed can be avoided.

Remaining difficulties in a more rigorous and complete morphological analysis of nucleoid structure arise from the peculiar dimensions of its basic structural element, the DNA molecule, and the moderate degree of regularity in geometrical arrangement which probably is lower than in chromosomal structures of higher organisms.

Despite these limitations, nucleoid structure at present is better understood than any chromosomal structure in higher organisms, and further progress is to be expected. This progress may consist in investigating a number of complete series of extremely thin sections at high magnifications and three-dimensional reconstruction of the pattern observed, or in certain types of experimental approach which may involve localization of sites of synthesis and replication of DNA by combination of cytochemical and electron-microscopic techniques.

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DISCUSSION

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In this discussion I shall continue from where Dr. Fuhs concluded and consider further the membrane or mesosomal attachment to the nuclear body. To begin with, I should like to disagree with Dr. Fuhs' closing comment, in that my work and that of other electron microscopists has suggested that mesosomes do play an active role in cell division.

When membranous organelles or mesosomes were first observed by several workers in sections of Ryter-Kellenberger fixed bacilli, their arrangement immediately suggested not only a possible role in separating the consistently replicating deoxyribonucleic acid (DNA) but also an implication in transverse septum formation. This latter implication was present in the earlier work of