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The Bacterial Nucleus: a History

THOMAS D. BROCK

Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin 53706

INTRODUCTION

The concept of the nucleus and its role in the hereditary continuity of the plant and animal cell was firmly established in the latter part of the 19th century (12), but it was not until the 1960s that the essential nature of the bacterial equivalent was discerned (7, 8). In actuality, there is no bacterial analog of the nucleus of higher organisms, but most workers who studied the question worked hard to find a bacterial parallel. Because of the central role of the chromosome in the life history of the higher organism and its clearly demonstrable role in heredity, most workers also sought diligently for a chromosome or chromosomes in bacteria. Indeed, even today the term *chromosome* is often used in bacteriology to refer to what is essentially a naked deoxyribonucleic acid (DNA) molecule (59). Current usage seems to assume an equivalence in bacteria between the terms *chromosome* and *nucleus*. Other terms used are *nuclear region*, *nuclear body*, *nucleoplasm*, and *nucleoid* (21). The latter term seems to be most widely used at present (30, 49).

The concept of the nucleus is essentially a morphological one, and one might have expected that the nature of the bacterial nucleus would eventually be discerned by sophisticated studies in electron microscopy. However, the real nature of the bacterial nucleus was uncovered not by morphological but by genetic studies. It was only after the essential nature of the genetic recombination process in bacteria had been worked out (by Hayes and Jacob and Wollman; see reference 23) and the process of DNA synthesis had been studied in growing cells (21) that intelligent research on the nature of the bacterial nucleus could be pursued. An analysis of how the bacterial nucleus was perceived also sheds considerable light on the role of precedent and preconception in the practice of biological research.

In addition to the genetic implications, ideas on the bacterial nucleus also influenced to a great extent concepts about the place of bacteria in the living world. Therefore, a second portion of the present paper deals with the various ideas about bacteriological classification. It is now common to classify the living world into two broad groups, the eucaryotes and the procaryotes, based on the nature of the nucleus. However, the distinction between eucaryote and procaryote (as well as the establishment of the terms themselves) was only made relatively recently (64). However, the existence of the term procaryote has confused an understanding of the essential nature of the bacterial nucleus. Although the term *procaryote* implies a structure that was a precursor to a true nucleus, current studies in molecular evolution provide no evidence that the procaryotes as we know them were forerunners of the eucaryotes. The most reasonable hypothesis, based on sequence analysis of ribosomal ribonucleic acid (RNA) molecules, is that both eucaryotes and procaryotes diverged early from a common ancestor (the so-called progenote) and that neither group is more ancient than the other (68).

THE EUCARYOTIC NUCLEUS

The history of the eucaryotic nucleus has been covered in detail in many sources (12, 47, 53, 60) and will be presented here only in outline form. The central body in cells was readily seen by microscopists of the early 19th century. The term *nucleus* itself was coined by the Scottish botanist Robert Brown in 1833 and used extensively by Mathias Schleiden and Theodor Schwann in their presentation of the cell theory. An important advance in cytological research came with the introduction in the 1870s by the Carl Zeiss Company of the Abbe condensor and the oil immersion lens, which made possible observation (and photography) of chromosomes. The continuity of the nucleus and its behavior during cell division were first clearly described by the plant cytologist Eduard Strasburger in 1875 to 1880. Strasburger showed that a new nucleus arises from a preexisting nucleus

by division, rather than de novo. In animals, the process of nuclear division was described by Walther Flemming in the same decade, and it was Flemming who coined the term *mitosis*. Strasburger coined the term *cytoplasm*, as well as terms for some of the key stages in the mitotic process: *prophase*, *metaphase*, and *anaphase*. DNA was first characterized by Friederich Miescher in 1869, who purified it from cell-free nuclei and termed it *nuclein*. The use of coal-tar dyes for staining cells and cell components was introduced by Paul Ehrlich in the decade of the 1870s and soon led to the visualization of chromosomes. Flemming coined the term *chromatin* for the characteristic staining material seen during the mitosis process, and the term *chromosome* was coined by W. Waldeyer in 1888 for the structure containing chromatin. By the turn of the century, the constancy of chromosome number and the behavior of chromosomes during the life cycle of the organism had been firmly established. E. B. Wilson, in his highly influential book (66), described the alternation of generations and the concept of reduction division. By the second decade of the 20th century, genes had been localized to chromosomes by Morgan and his school (43) and the role of chromosome pairing during genetic crossing over had been described.

The staining methods used to visualize the nucleus encompass several steps: (i) treating tissues with an appropriate fixative to preserve structure; (ii) staining the nucleic acid or protein or both with an appropriate dye; (iii) counterstaining other cellular constituents with a different dye to provide contrast. Because the nucleic acids are polyanions, any of a variety of basic (cationic) dyes will combine with them strongly. What is actually seen after the staining procedure will depend on the phase of cell division. In "resting" nuclei, the nuclear membrane is intact, whereas during mitosis this membrane breaks down, condensation takes place, and chromosomes can be seen. With appropriate material, it is possible to recognize individuality in the chromosomes and to track particular chromosomes from one division to the next and through the meiotic and fertilization processes.

Another staining technique which became widely influential (although ultimately of little utility in bacterial cytology) was the so-called nucleal reaction of Robert Feulgen, first reported in 1924. The Feulgen reaction was based on the observation that, when DNA was treated with acid, purine bases were split from the deoxyribose sugar, liberating aldehyde groups, and these aldehyde groups could be stained with the Schiff reagent. RNA is Feulgen negative. Under the controlled conditions of the Feulgen reaction, the phosphodiester backbone of the DNA remains intact, thus permitting localization of DNA within cellular structures. The Feulgen reaction became widely used in plant and animal cytology and provided strong evidence that DNA was restricted to nuclei and chromosomes.

BACTERIOLOGICAL BACKGROUND

During this exciting period for biological research, bacteriology was developing primarily as an applied science. Geneticists and cytologists studying higher organisms rarely studied bacteria, and the whole field of bacteriology developed, to a great extent, independently of the rest of biological science. Bacteriology as a discipline arose primarily out of medicine, mainly through the school of Robert Koch (3). Although certain structural features of bacteria, such as toxins and cellular antigens, were extensively studied, general aspects of bacterial physiology and cytology were pursued primarily in the context of methodology, since the key goal was to cultivate bacterial pathogens and characterize their behavior in the host and in the environment. (Another motivation for research on bacterial physiology, industrial microbiology, did not develop until after World War II, with the rise of the antibiotics industry.) A few biologically inclined bacteriologists studied general questions, but these were, with a few exceptions, not in the "mainstream" laboratories.

FIG. 1. Two cells of *Bacillus tumescens* with six nuclei. Drawn from a formaldehyde-fuchsin preparation. From Benecke (2).

Ideas about the bacterial nucleus at the peak of this "classical" period of bacteriological research (which was dominated by German scientists) are summarized in the influential textbook by Benecke (2): "The present situation on the nucleus question is as follows: A number of experienced bacteriologists believe that the bacterial cell is devoid of a nucleus, that it has neither a typical nucleus nor is there any evidence of any sort of morphological equivalent. They believe that structures that have been considered by others to be nuclei are something else, perhaps reserve materials. . . . Some researchers, on the other hand, believe that bacteria do possess a nucleus which differs from that of the higher fungi only in being smaller and because of this is visible only as small homogeneous particles, even when the best staining procedures are used." Figure 1 presents Benecke's cytological representation of the bacterial nucleus.

Even up to the end of World War II, cytological studies on bacteria presented, indeed, formidable obstacles. A bacterial cell is much smaller than the cell of a eucaryote. In fact, a bacterial cell is even smaller than the nucleus of many eucaryotes. It was not really until research on bacterial genetics became acceptable, after the work of Luria and Delbrück and Lederberg, and especially after the announcement in 1953 by Watson and Crick of the structure for DNA, that knowledge of the nature of the bacterial nucleus even became of interest to geneticists. Insight into pre-DNA pre-genetic ideas on the nature of the bacterial nucleus can be obtained from the textbook on bacterial cytology by Knaysi (31) and the highly influential and widely read book by Dubos (14). The Dubos book contained a widely cited addendum by C. F. Robinow on the bacterial nucleus (see below).

Many attempts were made to stain bacterial nuclei with procedures that worked with eucaryotes. However, bacteria presented formidable obstacles to cytologists: (i) small size (the nuclear structures of bacteria are near the limits of resolution of the light microscope); (ii) cytoplasmic RNA (ribosomes are distributed virtually uniformly throughout the bacterial cytoplasm, so that when a bacterial cell is stained with a basic dye, it gives a uniform appearance); (iii) volutin granules (many bacteria form polyphosphate [volutin] granules, which are of nuclear size and which react strongly with basic dyes); (iv) absence of a true nucleus (we now know that bacteria do not contain anything equivalent to the eucaryotic nucleus).

Procedures developed to overcome difficulties i, ii, and iii above can be briefly outlined. (i) To avoid the problem of small size, large bacteria were studied. As Knaysi (31, p. 82) explained, ". . . if the ratio between the size of the nucleus

and that of the cell in other microorganisms holds for bacteria, the dimensions of the nucleus of the bacterial cell would be in the neighborhood of the resolving power of the light microscope. It must be remembered, however, that there are bacteria with large cells. . . . [Thus] the demonstration of a nucleus in these [large] cells should be not only possible but easy." Indeed, many studies were done with large organisms such as *Bacillus megaterium*. (ii) Regarding masking by RNA, by the mid-1940s, it was accepted that cytoplasmic RNA masked nuclear staining. The most common procedure for overcoming this obstacle was removal of RNA with a brief acid treatment (for instance, 1 N HCl at 60°C for 10 min). When the enzyme ribonuclease became available, this was sometimes used instead of acid (with essentially equivalent results). (iii) Volutin granules could be reduced by partial starvation. Also, volutin differed in solubility from chromatin and did not show the Feulgen reaction.

Although the major difficulties could be overcome by use of the above procedure, not all workers were careful to apply them properly, so that many erroneous papers appeared in the literature. Thus, by the time of the review on bacterial cytology by Lewis (36) and Dubos' influential book (14), over 50 years of research had been carried out on the bacterial nucleus, by a wide variety of workers, using a wide variety of techniques in a wide range of organisms. It is not surprising that opinions varied markedly on the nature of the bacterial nucleus.

Following Lewis, Dubos lists no fewer than eight distinct theories regarding the nature of the bacterial nucleus, as follows.

(i) The bacteria do not possess a nucleus or its equivalent.

(ii) The cell is differentiated into a chromatin-containing central body and peripheral cytoplasm.

(iii) The bacterial body is a nucleus devoid of cytoplasm: a naked nucleus or nuclear cell.

(iv) The nucleus consists of several chromatin bodies, a chromidial system, scattered throughout the cytoplasm.

(v) The nucleus may occur as a discrete spherical body, an elongated chromatin thread, or scattered chromidia, depending on the stage of development. That is, bacteria have a polymorphic nucleus.

(vi) The nuclear substance consists of fine particles of chromatin dispersed uniformly in the cytoplasm but is not distinguishable as morphological units: a diffuse nucleus.

(vii) The protoplast contains one or more true vesicular nuclei.

(viii) The nucleus is a naked invisible gene string, or a chromatid-encrusted gene string analogous to a single chromosome.

Some of these theories are based on ideas that go back to the middle of the 19th century, whereas others are based on more recent ideas. Since the electron microscope had not yet become available, all ideas at this time were based on light microscopic studies. The following quotation from Dubos (14, p. 22–24) encapsulates ideas as they stood at the time of writing:

> It has been assumed that bacteria, standing at the threshold of organized living matter, are devoid of any structure suggesting a nucleus, and are therefore representatives of those cell-like organism which, because they are not nucleated, have been called cytodes by Haeckel. During the past few decades, however, attempts have been made to analyze, in terms of classical genetics, the process of transmission of hereditary factors in bacteria, and the mutation-like phenomena which they exhibit so frequently. Many have taken for granted that these phenomena occur through the agency of genes, organized as chromosomes in a nucleus, similar to the structure found in higher cells. . . . All the early claims of the existence of nuclei in bacteria rested upon the result of staining reactions due to the existence in these cells of certain substances—either diffuse in distribution or organized in the form of granules—which react toward the basic aniline dyes like the chromatin of higher organisms. Interpretations of these microscopic observations have ranged all the way from the hypothesis that bacteria are not nucleated, to the view that the entire bacterial cell is a nucleus, the cyoplasm being lacking or reduced to an invisible layer. . . . Finally, it has not yet been proven that the transmission of hereditary characters . . . obeys the mendelian laws and it is not possible therefore to argue from a similarity of biological behavior to an identity of cellular structure.

Dubos then discusses the immense technical difficulties in observing nuclei and chromosomes in cells as small as bacteria. One important concept that had already been established at the time of this writing was that the nucleus of the eucaryote was rich in nucleic acid of the deoxyribose type and that the Feulgen staining reaction was specific for DNA. Further, the seminal work by Avery et al. (1) on the chemical nature of the transforming principle had already been published (and was well known to Dubos since he was in Avery's laboratory during most of the time that this work was carried out [15]). Reviewing cytological work on bacteria, Dubos in his 1945 book (14, p. 29) concludes with the following key statement. "Granted that the transmission of hereditary characters in bacteria presents at least some analogy with the same process as it occurs in higher organisms, one may assume that this process takes place through the agency of genes. These genes then should maintain a fixed position in regard to each other; they should synchronize in division and be distributed in such a manner that a full complement of them could find its way into each daughter cell. A nucleus, reduced to the lowest essentials necessary to meet these requirements, could consist of a single gene string existing as a small granule or as a rod-like body rather than as a definite vesicle separating it from the cytoplasm."

The idea that the bacterial nucleus consisted of a single gene string sounds the most modern and perhaps closest to our current understanding. This theory was apparently first proposed by Carl C. Lindegren in 1935 (37). (Lindegren had come from the strong genetic school that Morgan had set up at the California Institute of Technology and had done pioneering work on *Neurospora* genetics. After the brief period of working on bacterial genetics described here, he turned to yeasts and spent the rest of his life carrying out fundamental [and frequently controversial] studies on yeast genetics.) According to Lindegren: "1. The gene is the fundamental particulate living unit and without it life is impossible. 2. Life of order of complexity higher than the gene is based on aggregation and mutational differentiation of genes. A linear aggregate of genes is the 'chromosome' and an aggregation of chromosomes is the 'nucleus' which governs cellular and metabiontic life. 3. The genes characterizing a given higher organism form a definite complement or set which must be present in its entirety to insure normal development of the organism. 4. The genes are arranged in the gene-strings in a certain definite order. 5. It is not the chromatin which is the essential hereditary material. The chromatin like most other protoplasmic constituents is merely produced by the action of the genes. The genes which are imbedded in the chromatin are the essential hereditary materials. . . . A nucleus is, therefore, defined as consisting of one more strings of genes maintained in a definite linear order and always present under normal conditions." Lindegren then proceeded to discuss the problem of satisfactory

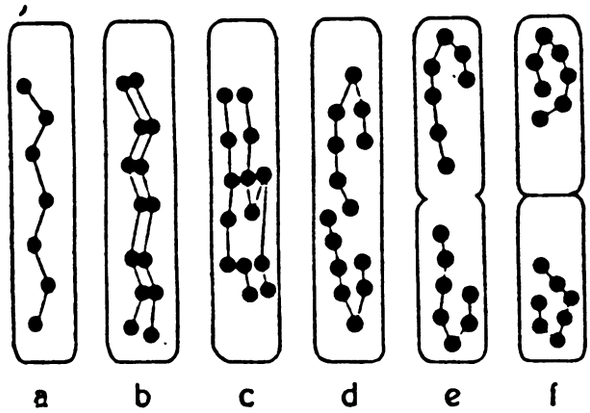

FIG. 2. Hypothetical stages in the division of a bacterium, as pictured by Lindegren (37). Each gene string is drawn to scale. (a) Initial state; (b) gene string splitting into two equal halves; (c and d) the split halves being pulled apart (spindle-fiber attachments were postulated); (e) the two gene strings in opposite halves of the cells; (f) transverse fission dividing the cell without disturbing the linear arrangement.

partioning of genes between daughter cells at cell division and concluded that some mechanism must exist to ensure this accurately. He concluded that a diffuse nucleus is impossible and that some mechanism must exist to keep all genes of a bacterial cell together during division. If a nucleus does not exist, then a naked gene string can be postulated which will ensure proper partioning. Although this theory sounds surprisingly modern, a close reading of Lindegren's papers shows that his idea only superficially resembles current understanding. At the time Lindegren was working, the dogma of classical genetics viewed the genes as particles arranged in a linear fashion ("beads on a string"), and Lindegren's hypothetical drawings of the partioning of the "gene string" during cell division (Fig. 2) clearly show this idea. That the chromosome and the gene string might be the same thing was not in Lindegren's thinking. And although even the idea of a naked gene string might have proved useful to those doing research on bacterial genetics, it was abandoned when C. F. Robinow's work (discussed below) provided such apparently strong evidence for the existence of a real bacterial equivalent of the nucleus of higher organisms. This is shown in the following quotation of Dubos (14, p. 28). "Although there exists some evidence for the view that the nucleus of bacteria consists of a single gene string, this hypothesis has probably become unnecessary in the light of the new information concerning the chromosome-like bodies which have been described. . . . Adaptation of the classical staining reactions to the study of bacterial cytology has provided such striking pictures of several morphological structures of the bacterial cell that one may expect great progress from these techniques in the near future."

Carl F. Robinow's work on the bacterial nucleus had been published during World War II in British journals, but became best known through Dubos' book (14), to which Robinow wrote an addendum. It is clear from a close reading of Robinow's work that his techniques were based strongly on those of Piekarski (50, 51), who had studied dividing chromatin bodies in bacteria by using ultraviolet microscopy as well as light microscopy with the Feulgen reaction. Piekarski had also demonstrated chromatin structures by Giemsa staining after acid treatment, and it was the latter procedure which Robinow systematically used in his work. This procedure, which came to be called Robinow's acid-Giemsa technique, had the advantage over the Feulgen reaction that it not only stained the "chromatinic" structures more deeply, but at the same time showed the outlines of the bacteria as well as their internal cell boundaries. This appeared important because it allowed a regular demonstration of chromatin bodies at any stage of the cell division cycle (44). But it was also clear that Robinow based his work on the preconceived idea that not only did bacteria have nuclei, but they also underwent a mitotic process. To Robinow, bacteria were no different than eucaryotic cells, only smaller. For instance, throughout his paper Robinow uses terms such as "nuclear structure," "chromatinic body," "nucleoid," and "chromosome." At one place, he describes a particular figure as demonstrating "telophase."

Robinow's basic cytological procedure began with a fixation of the bacterial cells with osmium tetroxide vapors, followed by treatment with 1 N HCl for 7 to 10 min at 60°C and then staining with Giemsa solution. The preparations were mounted under cover slips in water and viewed with an oil immersion lens, using a condensor of high numerical aperture. Robinow's cytological technique was superb, and he obtained beautiful photomicrographs of his preparations, many of which were published in Dubos' book. He was careful to use cultures whose growth cycle had been controlled, so that a reasonable correlation between the cell division process and nuclear arrangement (and division) was possible. Figure 3 shows a diagram Robinow published illustrating various stages of cellular and nuclear division.

In light of the careful electron microscope work of Kellenberger and co-workers (26), discussed below, it seems evident that Robinow's fixation procedure caused aggregation of the DNA into coarse clumps, each more or less corresponding to one DNA molecule. "Division figures" were observed when these coarse aggregations formed in various parts of the cell and at various stages of the cell division cycle. The DNA precipitates observed by light microscopy of whole cells could appear in any of a variety of arrangements, depending on the stage of division, the number of nucleoids per cell, the way the cell was disposed under the microscope, and the way the precipitation process had occurred. Robinow, of course, knew none of this, but interpreted his photomicrographs in terms of his preconceived idea that bacteria had nuclei composed of chromosomes and that these nuclei underwent a conventional mitotic process. He concluded that *Escherichia coli* had two pairs of chromosomes. Robinow's thinking can be illustrated by the following quotation: "The chromatinic elements have been seen in growing bacteria belonging to many and widely different species, in fact wherever they have been looked for. They divide by splitting lengthwise and are arranged in orderly configurations obeying simple numerical relationships, their division precedes cell division, they have many times been shown to give a clear-cut Feulgen reaction . . . and Giemsa solution stains them like chromatin. Jointly, these observations suggest very strongly that the chromatinic rodlets are integral constituents of the bacterial cell and that they may reasonably be regarded as the bacterial cell's equivalent of *chromosomes*" (italics in original). However, an examination of Robinow's figures shows that his chromosomes are not constant in morphology or in number per cell, as the chromosomes of higher organisms would be.

The influence of Robinow's work, especially for bacteriologists, was considerable. Thus, 20 years later one reads the following quotation in the most influential bacteriology

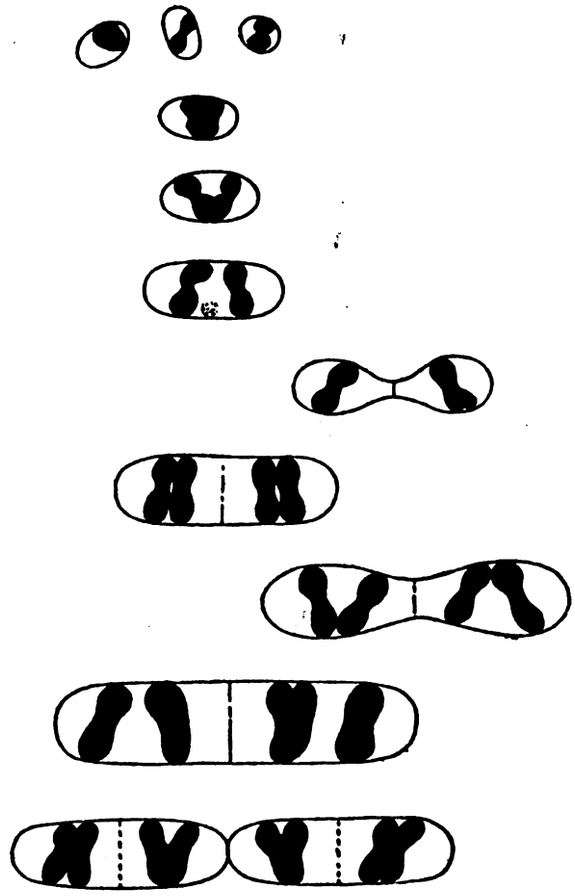

FIG. 3. Stages in cellular and nuclear division in *E. coli*. From Dubos (14); reproduced with permission from Jean Dubos.

textbook of its day: "The presence of nuclei in bacteria was established with certainty only about twenty years ago . . . all bacteria contain discrete, intracellular bodies with the chemical properties expected of nuclei which appear to divide in co-ordination with the division of the cell" (63, p. 384, 466). This statement is then supported with light photomicrographs supplied by C. F. Robinow. In the discussion of bacterial genetics later in this book, Stanier et al. return to the idea of the bacterial nucleus and make the following statement: "The DNA of procaryotic cells is localized in a discrete body called the 'nucleus'." Note that, when this textbook was published in 1963, Kellenberger's important work (discussed below) had been in print for at least 4 years, and Kellenberger had even published a well-documented review of his work in a widely distributed symposium of the Society for General Microbiology (24). Kellenberger (24, p. 53), for instance, makes this unequivocal statement: "The 'dense chromosome swimming in a nuclear sap' has to be considered a coagulation artefact." Kellenberger's work is discussed in detail later.

Another paper of this period that appeared to provide strong evidence for the bacterial nucleus was that of Mason and Powelson (41). These workers observed dividing bacteria with a phase microscope, suspending the cells in a high concentration of serum albumin so that the refractive index of the cytoplasm was the same as that of the surrounding medium. The gel-like DNA material, with refractive index different from the cytoplasm, appeared dark within a light cytoplasm and was thus naturally interpreted as nucleus. With this technique, the bacterial "nuclear bodies" could be readily observed in the living state and their behavior during division could be studied. The photomicrographs obtained by this technique were remarkably similar to those obtained with the Robinow technique, and since no fixation or staining was used, it was natural to conclude that the observed nuclear bodies were real. Although the correspondence between the Mason-Powelson technique and the Robinow technique was merely fortuitous, it led to confusion for almost a decade of research on the bacterial nucleus.

GENETICS RESEARCH AND ITS IMPLICATIONS FOR UNDERSTANDING THE BACTERIAL NUCLEUS

Although studies on the bacterial nucleus were generally included as part of the broader question of bacterial cytology, it was clear that most workers studying the nucleus had genetics in mind. In his widely read book on bacterial cytology, Knaysi (31) introduces his chapter on the nucleus with the following sentences. "The bacterial cell possesses a mechanism of inheritance which, in other cells, is believed to be largely a function of the nucleus. This experimental fact is tacitly admitted by all bacteriologists when they transfer a culture of bacteria and expect the development of a strain similar to the parent culture."

Ultimately, it was not cytology but genetics which led to an understanding of the bacterial nucleus. Bacterial genetics, however, did not develop until over 50 years after the first cytological studies on the bacterial nucleus. Although mutation as a mechanism for genetic variation in bacteria had been studied for many years, it was the seminal paper by Luria and Delbrück (39) that initiated modern research in this area. About the same time, the long-term project by Oswald T. Avery on the nature of the transforming principle was coming to completion (1), focussing attention on DNA and its arrangement in the cell. Soon thereafter, Joshua Lederberg described a mechanism of genetic exchange in *E. coli* K-12 (33).

Lederberg initially interpreted his work in terms of cellular fusion followed by nuclear fusion and crossing over during meiotic reduction of the zygote. Although this hypothesis was the simplest which corresponded with the genetic facts, it proved to be erroneous (see Jacob and Wollman [23, p. 16–41] for a review of this early work). Undoubtedly, Lederberg's hypothesis of cell and nuclear fusion served to mold cytological opinion around the equivalence between the bacterial nucleus and that of higher organisms. Another study that seemed to agree with this was that of Witkin (67). Using Robinow's staining procedure, Witkin studied partioning of nuclei during cell division after mutagenesis and related this to the segregation of genes. Essentially, Witkin was studying the segregation of genetically distinct DNA molecules from cells containing more than one DNA copy (interpreted as multinucleate cells). Although Witkin's work was well done (and is actually still of practical relevance in mutation studies on bacteria), it was used for many years to support an incorrect model for the bacterial nucleus.

However, it soon became clear that Lederberg's model of cellular fusion followed by nuclear fusion and reduction division was incorrect. In a study of far-reaching importance, Hayes (20) showed that genetic exchange in *E. coli* K-12 was unidirectional and that mating strains could be divided into two classes, donors and recipients. This observation, and the discovery of the fertility factor F by Lederberg and co-workers at about the same time, led to the

discovery of strains that underwent genetic recombination at high frequency (Hfr). By the use of these Hfr strains, the basic mechanism of gene transfer was worked out by Hayes and Jacob and Wollman. Three aspects of this genetic exchange are relevant here: (i) one-way transfer of genetic material from donor (Hfr or F^+) to recipient (F^-); (ii) partial transfer of genetic material; and (iii) transfer in an ordered manner, so that interruption of conjugation (by agitation of the mating culture) blocked the entry of some genes without affecting entry of other genes. The latter observation led to the use of the interrupted mating technique in genetic mapping. It also led to the hypothesis of the circular chromosome (23). Although the concept of the circular chromosome was initially just a genetic formalism, research from a number of laboratories (especially that of Cairns [7, 8]) soon gave it physical reality. Thus, it was the genetic work that demonstrated the fallacy of equating the bacterial nucleus with the nucleus of higher organisms and focused attention on research that cast doubt on the accepted doctrine.

DNA SYNTHESIS AND CELL DIVISION

Another type of study carried on in the 1950s and early 1960s, mainly in the laboratory of O. Maaloe, involved measurements of DNA synthesis during the bacterial growth process. These studies, reviewed by Maaloe (40) and Schaechter (56), showed that, in contrast to eucaryotes, DNA synthesis in bacteria was continuous throughout virtually the whole cell division cycle. This provided additional evidence that a conventional mitosis process was not present in bacteria. Lark et al. (32) attempted to correlate the nucleus (detected by a modification of the Robinow technique) with cell division, using synchronized cultures. This work was extended by Schaechter et al. (57, 58) with measurements of DNA and RNA. The number of nuclei per cell was shown to depend on the growth rate, rapidly growing cells having more nuclei than slow-growing ones. All of this work culminated in the important study by Cooper and Helmstetter (13) on chromosome replication and the division cycle. By the time of the Cooper-Helmstetter work, the Cairns model of the bacterial chromosome was established (see later), and Cooper and Helmstetter concluded that the bacterial chromosome was equivalent to the nucleus.

ELECTRON MICROSCOPY OF BACTERIAL NUCLEI

As had been emphasized by bacterial cytologists (for instance, Knaysi [31]), the light microscope did not provide sufficient resolution for critical studies of the bacterial nucleus. When the electron microscope became available, it was natural that researchers would turn to this important tool to study bacterial cytology. Initially, observations were made of whole cells, but it soon became clear that, although bacteria were too small to be observed well in the light microscope, they were too thick to be observed well in the electron microscope. It was only after the technique of thin sectioning was developed that serious study of bacterial cytology became possible.

The thin section technique was developed independently by J. Hillier at the RCA Research Laboratories in Princeton, N.J. (9), and by Keith Porter and J. Blum at Rockefeller Institute for Medical Research in New York (52). The Porter-Blum microtome soon became a widely available commercial device, and the thin section technique rapidly became a standard procedure in bacterial cytology.

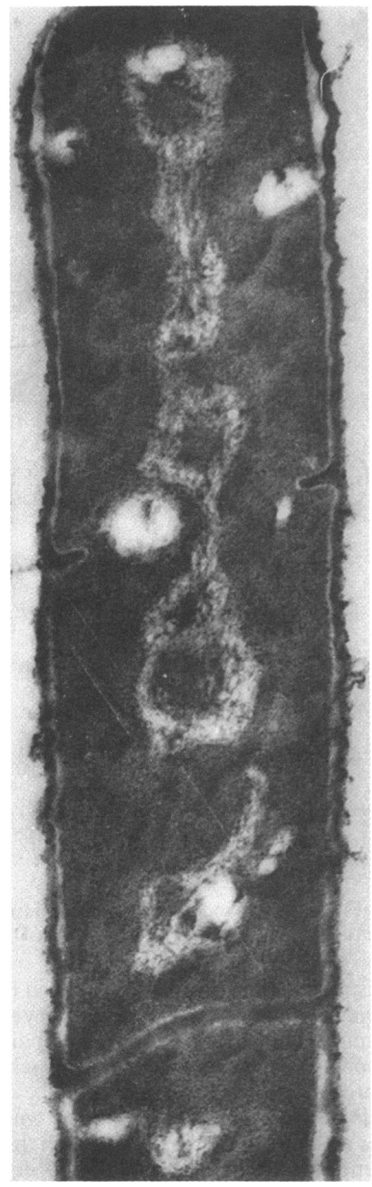

FIG. 4. Thin section of *Bacillus cereus* showing nuclear region and the absence of a nuclear membrane. From Chapman and Hillier (9).

The first electron micrographs of thin sections of bacteria were published by Chapman and Hillier (9). A typical photomicrograph is shown in Fig. 4. A major advance in bacterial cytology, these micrographs showed clearly that there was no obvious nuclear membrane: ". . . nuclear sites have a different texture than the cytoplasm, being almost entirely fibrous in character." Such a view did not agree with the concept of a nucleus which had developed with the Robinow technique.

A major advance in understanding the nature of the bacterial nucleus came from the work of Edouard Kellenberger and Antoinette Ryter. Motivated by a desire to understand the nature of DNA-containing plasms in bacteriophage-infected cells, these authors also carried out extensive studies on uninfected cells in various physiological

states. By the time Kellenberger and Ryter initiated their work, it was widely accepted that there were functional (and probably structural) relationships between the DNA of bacterial viruses and the DNA of bacterial cells. Kellenberger and Ryter used electron microscopy to study the bacteriophage replication process. During the phage replication process there is a breakdown of the bacterial nucleus, and the bacterial DNA is hydrolyzed and recycled into phage DNA. A pool of phage DNA develops within a few minutes of infection and later becomes packaged into mature virus particles. Kellenberger and Ryter developed preparation techniques that permitted preservation of the phage DNA in an unaltered state for electron microscopy. They showed that pH, divalent cations, and other solutes present in the medium during the fixation process, as well as the conditions of the embedding process, strongly influenced the appearance of the bacterial DNA in the thin sections. (A major advance was the development of Vestopal as an embedding plastic in place of methacrylate [26].) By making a systematic study of the conditions of fixation and embedding, they developed standard procedures (the so-called RK conditions) that preserved the DNA in a fine-stranded fibrillar state (26, 55). A typical electron micrograph obtained with these procedures is shown in Fig. 5. In these papers the term *nucleoplasm* or *nucleoid* was used to refer to the region under study. Kellenberger et al. carefully showed that the coarse coagulation of DNA-containing plasms seen by many workers was a fixation artifact. Considering the implications of their observations, Kellenberger et al. (26) made the following statement. "All evidence is now against complicated, complete mitosis in bacteria, i.e. morphologically the homogeneous nucleoplasm, and chemically the continuous production of DNA [during growth]. . . . These facts lead us to postulate that the genetic material of bacteria may multiply following the same mechanism as phage. The nucleoid would be simply a pool containing one or several bacterial genomes in a form similar to vegetative phage. To explain the genetic continuity of the bacteria . . . we have to assume the existence either of only one linkage group, or, when more linkage groups exist, of a high number of identical strands. . . . Nothing then prevents us from considering the bacterial nucleoid as one single multistranded chromosome. The main difference between chromosomes would be in the organization and the moiety which is not DNA." Or, in another place: "Our mind is still a prisoner of the eloquent picture of the mitotic cycle. Between this and the DNA molecule, however, there is a very great gap which we have to fill in the coming years" (24).

However, bacteriologists were reluctant to accept Kellenberger's idea. For instance, several years after Kellenberger's paper, in an influential review on the cytology of bacteria, Murray (44, p. 89) made the following statements. "The trouble with the soft outlines and almost homogeneously reticular interiors shown by Kellenberger is that they do not permit us to visualize the patterns of chromatin concentration that we must consider to be present from our light microscope studies. Either the total outline has been changed in the process of preparation, or the internal arrangement of DNA is not visible to us or has been deranged. For that matter one must agree with Kellenberger that the vacuoles and cords, so commonly represented, are not at all satisfying or convincing. But one cannot cast out the best of methacrylate embeddings; let us see what we can learn from them." In another review published 2 years later, Murray (45, p. 124, 125) was still writing about chromatin bodies, but had softened his objections to Kellenberger's work. Now we

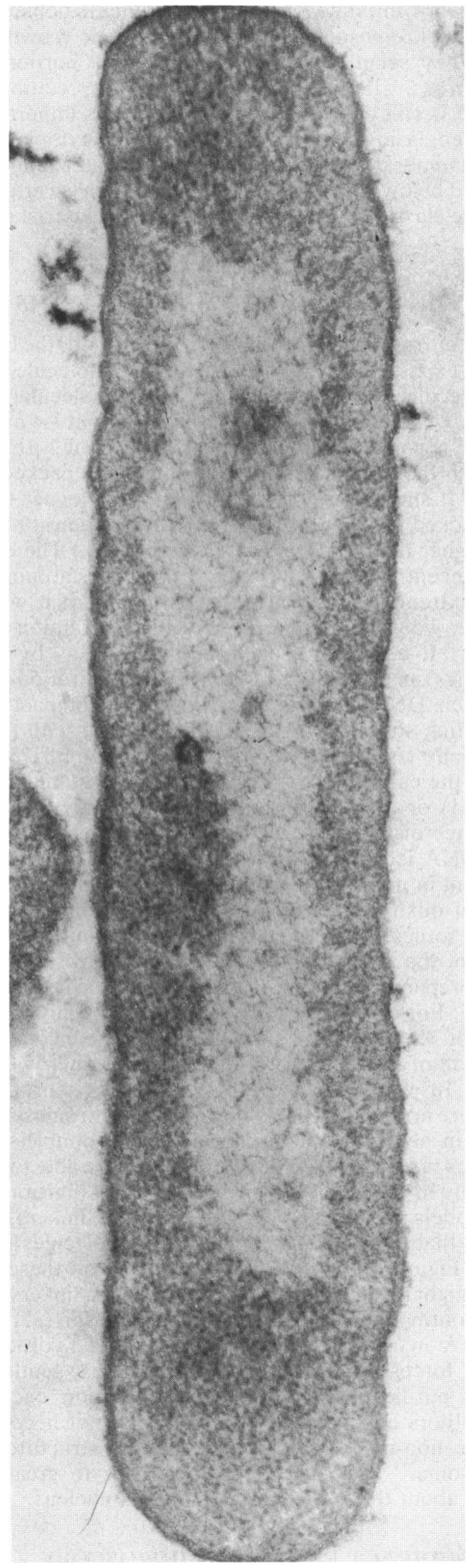

FIG. 5. Thin section of *B. megaterium* fixed and stained under conditions that preserved the structure of the nuclear region. Note that the nucleoplasm is composed of fine filaments and there is no nuclear membrane. From Ryter et al. (55); reproduced with permission from Zeitschrift der Naturforschung.

find the following statements. "The chromatin bodies are not obviously chromosomal at any time in their growth cycle. Indeed they seem to ramify in the central portion of the cytoplasm. . . . It matters little that the interpretation of fine structure is still open to debate . . . but it is important that the nucleoplasm is not bounded from the cytoplasm by a nuclear membrane." The works of Cairns and Kleinschmidt, discussed below, would soon put an end to these attempts to retain the classical Robinow picture of the bacterial nucleus.

MOLECULAR WEIGHT OF PROCARYOTIC DNA AND THE QUESTION OF PROTEIN LINKERS

As we currently understand it (25, 30, 49, 69), the bacterial genome exists as a single circular DNA molecule of very high molecular weight (4×10^6 base pairs; molecular weight, 4×10^9), whose total length (in *E. coli*) is about 1.4 mm. This molecule is packed into the interior of a cell of 2-μm length. (Although the bacterial genome is somewhat packed within the cell, it should be noted that it is much looser than the DNA packed in a phage head, which has a density 30 to 60 times higher than the bacterial nucleoid [25].) The effective DNA concentration in a typical growing cell is around 17 mg/ml, an extremely high concentration which, if it were in a test tube, would result in a virtual gel-like solution of great viscosity. If cells are opened gently (such as by lysis of protoplasts) in the presence of high concentrations of cations, each DNA molecule remains as a compact rapidly sedimenting structure that has been equated with the morphologically visualized bacterial nucleoid (49, p. 178). However, in the early attempts to isolate bacterial nuclei, either intact (61) or as solutions for chemical study, the critical importance of high cation concentrations was not realized. When DNA is released from cells at the cation concentrations used in most conventional buffers, the long molecules that spill out into the medium are extremely sensitive to shearing forces (35) and readily fragment. Thus, in the early studies of the chemistry and molecular weight of bacterial DNA, preparations of fairly low molecular weight were obtained. For instance, in the classical and highly influential studies of Meselson and Stahl (42) on the semiconservative replication of DNA, preparations with molecular weights of only 7×10^6 were used. To explain how such short pieces of DNA were arranged in the whole bacterial chromosome (and to explain also how such extremely long double-stranded molecules replicated, since they would not be able to unwind effectively in the time available during a cell division cycle), most models postulated the existence of "linkers," small proteins that connected the short DNA molecules together (17, 24). Figure 6 illustrates one concept of how these protein linkers might be disposed. The idea of protein linkers quickly became outmoded when Levinthal and Davison (35) showed that DNA in solution is subject to drastic hydrodynamic shearing forces, so that even under the most gentle conditions of purification extensive fragmentation occurs. As those authors conclude: ". . . it is clearly not necessary to postulate non-nucleic acid linkers in the structure of the chromosome." This important study was to greatly alter thinking about the nature of the bacterial nucleus.

DIRECT ELECTRON MICROSCOPY OF ISOLATED DNA

Another approach which was to have marked impact on understanding of the bacterial nucleus was that of Kleinschmidt and colleagues (28, 29) on the electron microscopy

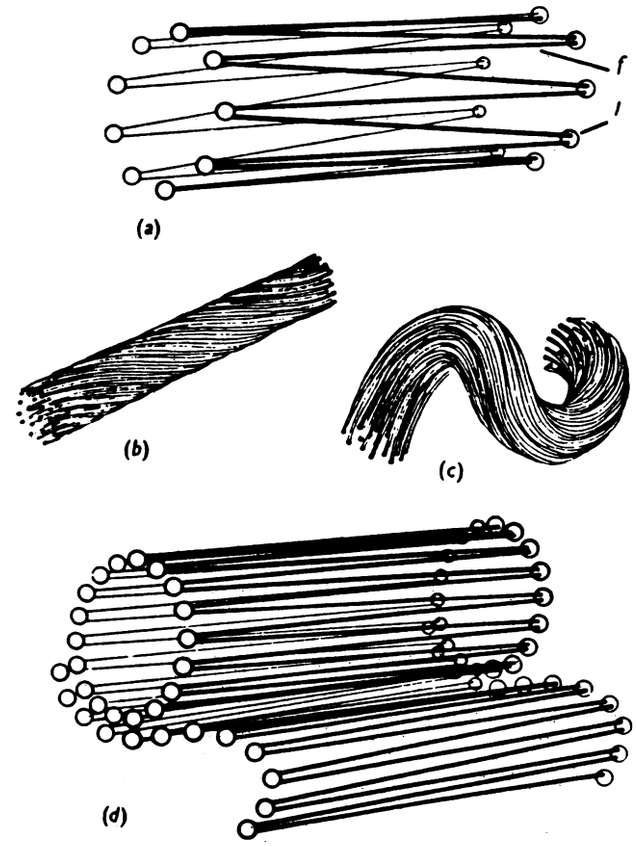

FIG. 6. Model of a bacterial nucleoid. (a) Schematic drawing: (f) represents the chromatin fibrils; (l) represents the linkers joining the fibrils. The linkers were postulated to provide bonds with a high degree of mobility, but the whole array was continuous, providing for a genetically circular linkage map. (b) In the real nucleus, the fibrils form a sort of rope. (c) This rope may form helices. (d) A proposed replication mechanism. From Kellenberger (24); reproduced with permission from the Society for General Microbiology.

of isolated DNA. In the Kleinschmidt technique (as it came to be called), cells or viruses are gently lysed in place on monolayers of protein-salt solutions, and the resulting films are picked up onto electron microscope grids. After shadowing with a heavy metal, the preparations are examined at high magnification. The images obtained by Kleinschmidt revealed extremely long molecules of uniform diameter, with no branches and very few free ends. The picture that the Kleinschmidt technique revealed (Fig. 7) was of extremely long threadlike structures protruding from the remains of the lysed cells that remained. Relating these images to the electron micrographs of thin sections obtained by Kellenberger and Ryter (discussed earlier), Kleinschmidt and Lang (27) concluded: ". . . the whole DNA content is present in undivided filamentous forms. Depending on the bacterium, these consist of one or only a few very long structures. The DNA filament is arranged in the cell interior as a yarn-like form which can be called 'nucleoplasm' " (translated from the German). (Parenthetically, the Kleinschmidt technique was to have implications considerably beyond its use to study the arrangement of DNA in the bacterial nucleus. It provided an important tool for studying the detailed structure of DNA molecules and showed the presence of circular and supercoiled plasmids, loops, replicating forms, and many other forms of DNA.)

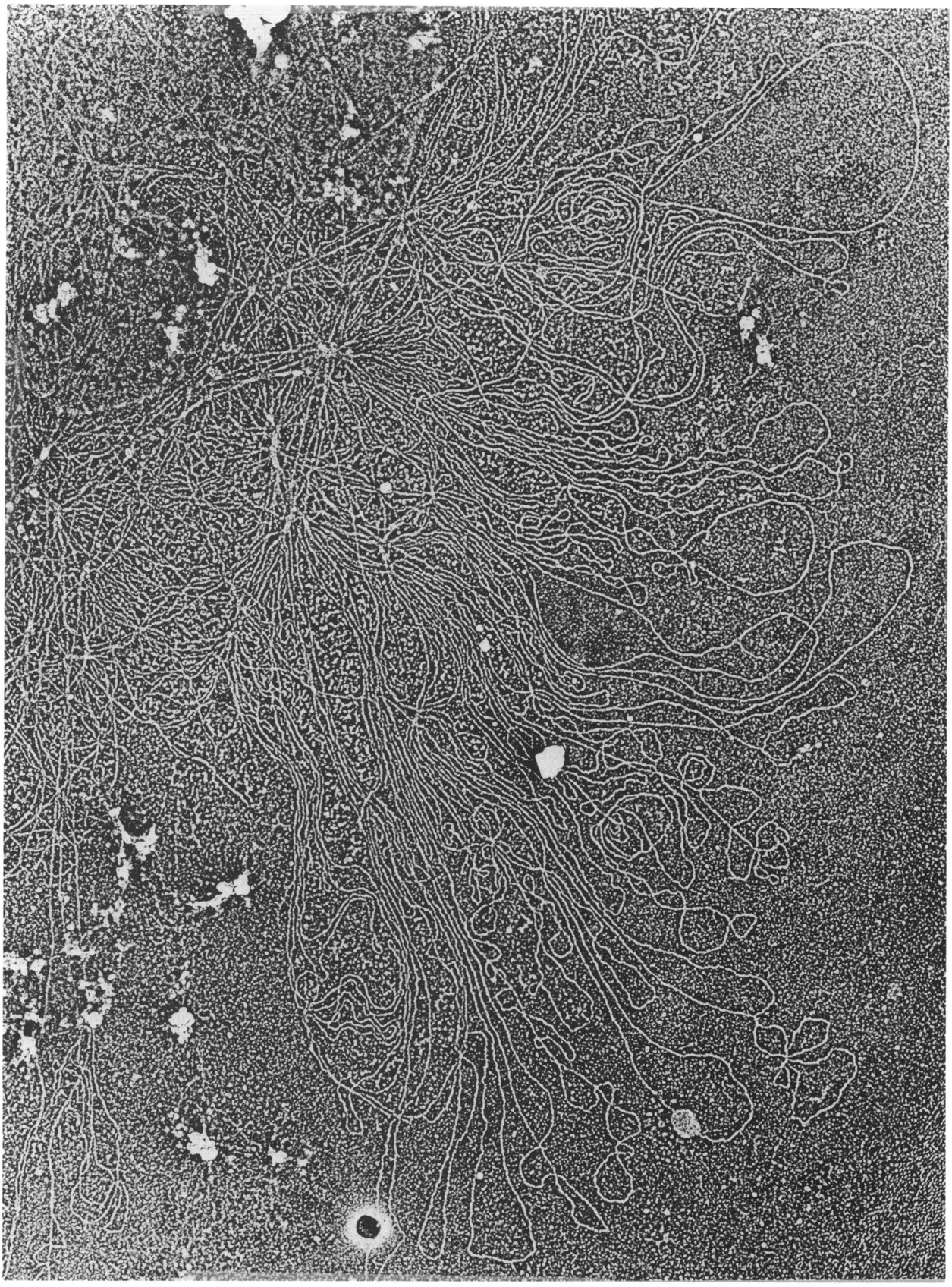

FIG. 7. DNA filaments of *Micrococcus lysodeikticus* spread out on a proteinaceous fibrin from a lysed protoplast. From Kleinschmidt et al. (28); reproduced with permission from Zeitschrift der Naturforschung.

AUTORADIOGRAPHIC STUDIES ON DNA

At the same time that Kleinschmidt's work was suggesting that the nucleus was a single long DNA molecule, John Cairns (independently of Kleinschmidt) was using an autoradiographic technique to measure the lengths of DNA molecules. This work not only provided an independent confirmation of Kleinschmidt's conclusions, but permitted a study of the cellular DNA replication process itself.

As a result of the development of the hydrogen bomb in the 1950s, huge quantities of tritium became available commercially, and a variety of tritiated biochemicals could be made. Among these, the most important was tritiated thymidine ([^{3}H]thymidine), which could be produced at extremely high specific radioactivity. Because of the low energy of the beta ray resulting from tritium decay, the mean range in an autoradiographic emulsion of a tritium beta track was <1 μm, permitting effective localization of the tritium tracer. With the high specific activity available (10 Ci/mmol), one disintegration occurred per micrometer of double helix per week, so that with fully labeled DNA a near-continuous line of grains along its length would result after a few weeks of exposure to the photographic film. Cairns developed a procedure for spreading DNA on films and bringing it into contact with the photographic emulsion for autoradiography.

In his first work, Cairns (5) used the autoradiographic technique to estimate the length of the DNA of bacteriophage T2 (released by gentle osmotic shock). The length obtained, 52 μm, agreed with the molecular weight of T2 DNA. Cairns then proceeded to a determination of the molecular weight of *E. coli* DNA, a much more difficult problem (6). Knowing from the work of Levinthal and Davison (35) that lengthy DNA molecules are subject to extensive hydrodynamic shear, Cairns handled his DNA carefully. The lengths obtained in the first work were 400 μm, somewhat shorter than reality, but much longer than the lengths calculated from the earlier molecular weight determinations of Meselson and Stahl (42). Cairns (6, p. 409) concluded: "Until the existence in DNA of non-nucleic acid links has been demonstrated, it is probably legitimate to think of these threads as molecules." Cairns then added in a footnote: "Since this paper was written, Kleinschmidt, Lang and Zahn have produced beautiful electron micrographs showing that protoplasts of *M. lysodeikticus*, lysed at an air-water interface, release their deoxyribonucleoprotein in the form of a tangled skein which has no visible free ends. Thus two dissimilar procedures suggest that bacterial DNA may exist as a single molecule."

Using an improved version of his autoradiography procedure, Cairns then proceeded to study the replication process of the bacterial chromosome itself. This work was published shortly after the circular chromosome model of Jacob and Wollman had been developed from genetic studies and provided a dramatic confirmation of the Jacob-Wollman model. Cairns' classic image, which was to appear in numerous textbooks, is shown in Fig. 8 (7). The total length of the molecule was now found to be at least 1,100 μm, equivalent to a molecular weight of 2.8×10^9. Although slightly lower than the molecular weight calculated from the DNA content per cell nucleoid, this value was close enough (given the vagaries of the spreading technique) to make it seem likely that the complete *E. coli* genome was in one circular molecule.

Cairns was also able to confirm that the DNA replication process began at a single point and moves bidirectionally around the circle. The Cairns model for the replication process is shown in Fig. 9 (Fig. 1 in reference 7). Although the molecular details of this process, especially how the double helix unwinds, had to await studies on the biochemistry of DNA replication (and especially the discovery of enzymes involved in winding and unwinding DNA), the overall model that Cairns presented in 1963 is still the accepted model today.

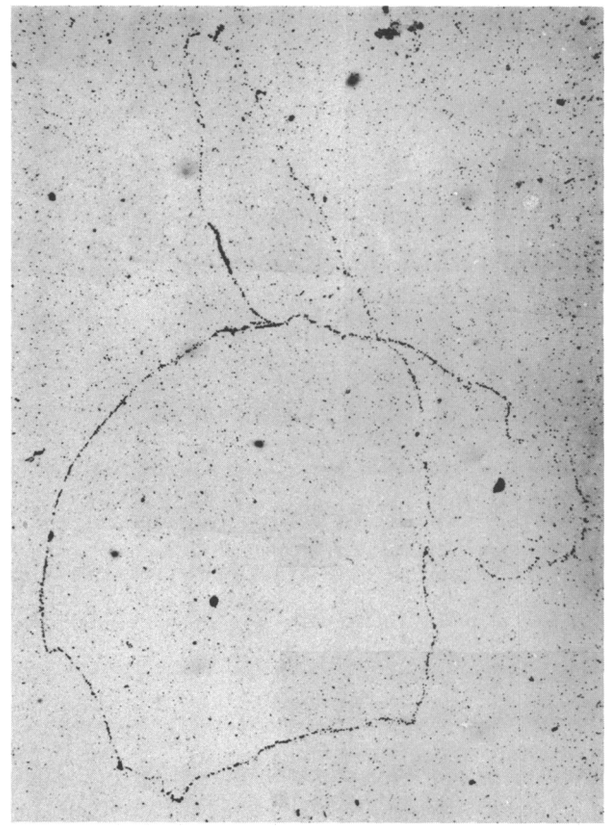

FIG. 8. Autoradiograph of the circular DNA of *E. coli* labeled with initiated thymidine for two generations. From Cairns (7); reproduced with permission from Cold Spring Harbor Laboratory.

A connection of the Cairns model with the morphological nucleus and the cell division cycle was made by Cooper and Helmstetter (13) in their work on chromosome replication during synchronized growth. To explain the increase in number of nuclei with increase in growth rate, Cooper and Helmstetter developed a model in which the replicating genome folded into dense masses prior to completion of a round of replication. This model (Fig. 10) relates the cytological observations of Robinow and later workers with the molecular studies of Cairns.

ASSOCIATION WITH THE MEMBRANE

An important hypothesis in models of DNA replication during cell division was an association of at least one point on the DNA molecule with the membrane, so that partitioning of duplicated DNA molecules could occur during division. The suggestion of a membrane association, first put forward by Jacob et al. (22), has seemed so right on theoretical grounds that it is generally accepted, even though the evidence to support it is fairly circumstantial (34, 48).

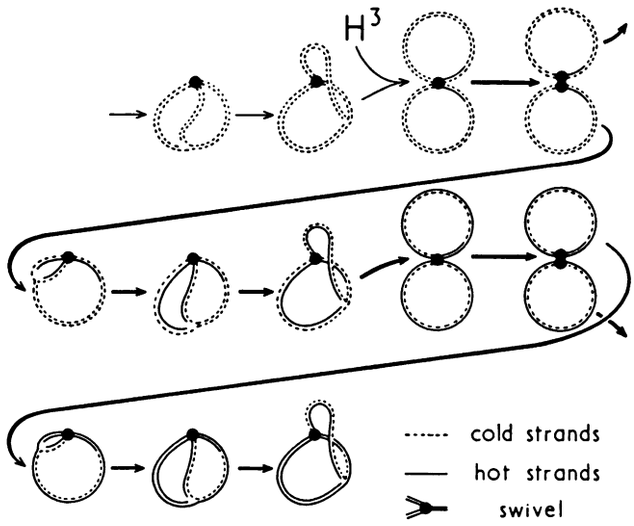

FIG. 9. Postulated model for replication of a DNA circle, based on the assumption that replication always begins at the same place and proceeds bidirectionally. From Cairns (7); reproduced with permission from Cold Spring Harbor Laboratory.

THE NUCLEUS AND GENE EXPRESSION

The physical organization of the DNA in the bacterial cell must influence the manner in which gene expression occurs. It is known (21) that messenger RNA synthesis is essentially continuous and that a single open reading frame such as the lactose operon is accessible to transcription throughout the whole division cycle. Because of the tight packing of DNA, numerous loops of supercoiled DNA occur, the neighboring duplexes being about 3 to 5 nm apart. Although thermal agitation is probably sufficient to keep genes accessible to the cytoplasm, it should be noted that in bacteria transcription and translation are linked, so that polysomes must be attached to the DNA via a messenger RNA link. There are a few studies (reviewed by Schmid [59]) suggesting that the state of the bacterial DNA can alter gene expression, with quiescent (untranscribed) genomes coexisting beside active genomes in the same cell.

OVERVIEW OF THE STUDIES CITED

At this point, I can present a brief summary of current understanding in the light of historical developments (see Schmid [59] for a recent review).

(i) The DNA of the bacterial genome exists as a long fibrillar or threadlike molecule that is more or less confined to a single region of the cell.

(ii) Although there are minor amounts of DNA-binding proteins, for the most part the bacterial DNA is present as a naked DNA molecule. There is no nuclear membrane.

(iii) DNA synthesis occurs throughout most of the cell division cycle, and partioning of replicated DNA between two daughter cells occurs via a site on the DNA which is probably attached to the cytoplasmic membrane.

(iv) Fixation and staining procedures cause the DNA to condense or aggregate, so that images seen in the light microscope resemble a nucleus. The nucleus, in the way in which the term is conventionally used, is thus an experimental artefact.

(v) The artefactual nucleus formed as a result of cytological treatments segregates as the DNA segregates, giving an appearance of nuclear division. Under some conditions, coagulation of the DNA into separate regions results in the appearance of images that can be interpreted as chromosomes.

(vi) It thus appeared to bacterial cytologists that a nucleus existed, even though one did not exist. Whether one "believed" in the existence of this nucleus depended upon one's prejudices and predilections.

(vii) Confusion existed (and still does, to some extent) between the process of DNA replication and the process of DNA partitioning. Descriptions of nuclear division are actually descriptions of the partitioning process.

THE NUCLEUS AND CLASSIFICATION OF BACTERIA

It is always tempting to derive classifications of organisms, but unfortunately these classifications are generally based on incomplete and often contradictory information. Nowhere is this statement more clearly supported than in attempts to use the nature of the nucleus in the classification of the bacteria.

Early ideas of bacterial classification have been nicely covered in Bulloch (4, p. 171–206). Since Linnaeus, a two-kingdom classification of living organisms had been used, with an organism being classified as either a plant or an animal. Based on the observation that bacteria moved under their own power, bacteria were placed with the animals. Antonie van Leeuwenhoek used the term *animalcules*, and the major workers from the 18th through the middle of the 19th century (Otto F. Müller, Christian G. Ehrenberg, and Félix Dujardin) followed this lead. It was Ferdinand Cohn (11) who first classified the bacteria with the plants. However, he realized the difficulty of encompassing microorganisms under the two-kingdom system, as shown by the following quotation (11, p. 5–9). "The simpler the organism, the fewer characteristics that are available for classification as either a plant or animal. The infusion animals that lack muscles and nerves and circulatory and respiratory systems are only imperfectly developed. On the other hand, a num-

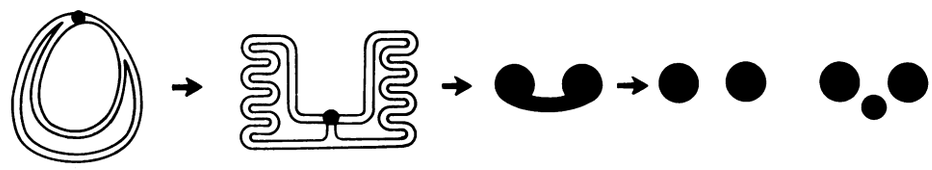

FIG. 10. Production of two "observable" nuclei from one "true" nucleus. The replicating genome (a) folds in dense masses, shown schematically (b), prior to completion of a round of replication. In the absence of artifacts, a dumbbell-shaped nucleus would be observed. (c) Artifacts occasionally obscure the connecting strand, resulting in two observable (d) or triple (e) nuclei. Redrawn from Fig. 7 of Cooper and Helmstetter (13), reproduced with permission from Academic Press, Inc.

ber of microscopic plants show independent motility, and even organs of motility such as we generally associate with organisms of the animal world. Indeed, in the very simplest forms of life the characteristics of the plant and animal kingdoms seem to be melded, so that the researcher experiences considerable doubt in deciding in which kingdom a given organism should be placed. We call the smallest, simplest, and lowest of all living organisms *bacteria*; they form the very boundary of life itself. On the far side of the bacteria no life at all exists, so far as our present-day microscopes can discern. . . . Almost all the older researches have classified the bacteria with the animals, because they possessed the property of independent motion. . . . [However,] when we consider the whole group of bacteria, there is no doubt that bacteria belong to the plant kingdom, in close relationship with the Oscillarias [blue-green algae or cyanobacteria]. In the bacteria, the motile stage alternates with a nonmotile one, the latter of which cannot be distinguished from a plant cell." Another property that led Cohn to classify bacteria with the plants was their mode of cell division, by simple cross wall formation. The presence of a wall was, for Cohn, a strong reason to classify bacteria with the plants (since animals do not have walls).

We should mention here one curious point: although classification of bacteria has presented great difficulties, it is rarely difficult for an experienced microscopist to distinguish a bacterium from a higher life form. For instance: "The most remarkable feature [regarding bacterial classification] . . . is that there has been so very little argument about the assignment of any particular organism or group of organisms to the bacteria." (64). How does one recognize a bacterium so easily: partly by size and partly by the absence of discernible cellular structure. Bacteria are so much smaller than other microorganisms and show such a lack of intracellular differentiation that even a relatively untrained microscopist can distinguish a bacterium from another microorganism. (The same cannot be said for distinguishing the other major group of nonnuclear organisms, the cyanobacteria. Cyanobacteria are generally much larger than bacteria, and the simple criterion of size is not sufficient. However, a competent phycologist never has any trouble distinguishing a cyanobacterium from an alga, because the latter show marked intracellular differentiation, primarily the noticeable presence of chloroplasts and cytoplasmic movement, absent from the cyanobacteria. But note that it is the chloroplast rather than the nucleus that serves in practice as the distinguishing characteristic between an alga and a cyanobacterium.)

As discussed in detail by Whittaker (65), the two-kingdom concept of classification had been questioned since the early 19th century, the major difficulty being what to do with the large number of microorganisms, primarily algae and protozoa, that seemed to represent intergradations between plants and animals. The great German biologist Ernst Haeckel proposed a separation of the lower organisms into a separate kingdom, Protista, which included all unicellular and unicellular-colonial organisms which formed no tissues (18). As a group, these organisms had earlier been called Infusoria. As Haeckel noted, Ehrenberg, in his influential work, concluded that the Infusoria, although tiny, were "perfect" organisms, with nerves, muscles, and other organs characteristic of higher organisms. However, improved microscopic studies had shown this idea to be false. According to Haeckel, the Infusoria were really organisms that consisted of single cells, and this idea had implications not only for the general theory of the cell, but for evolution. To Haeckel, the Infusoria were the lowest forms of life and, because of their "imperfect" nature, could be considered representatives of "original life forms." To reflect this idea, Haeckel (18) proposed that this group be called the Protista, the name Infusoria thus being reserved only for the protozoa: ". . . many infusoria are considered animals by zoologists, and plants by botanists, whereas others are claimed by neither, and others live sometimes like animals, other times like plants. . . . In order to get around these difficulties and develop a reasonable classification of organic life, [we] create a third independent kingdom of elementary organisms. This is our kingdom of *Protista* . . . the kingdom of neutral primitive beings." Descending even further on the evolutionary scale, Haeckel reaches the bacteria. "Finally, we find ourselves at the bottom step, with those extremely simple, marvellous beings, from which all organic life forms arose. . . . [These are] the single-celled protista, which we call *Monera*. . . . The Monera are truly organisms without organs. Their whole living body even when fully developed consists of only a simple glob of protoplasm, which in opposition to true cells even lacks a nucleus. . . . They multiply in a simple manner by division. It is of great theoretical interest that they represent an early stage in the development of life on our earth. Because only the Monera could have developed at the beginning by spontaneous generation; only Monera can be the oldest representatives of all other organisms." Because nuclei could not be seen in these cells, Haeckel concluded that they were not true cells, but nucleus-free structures that he called cytodes. Although this classification sounds reasonably modern, it is clear from a reading of Haeckel's work that with his primitive and careless microscopy he was incapable of determining properly whether an organism did or did not have a nucleus. Within the Monera, Haeckel placed a variety of protozoa that had an indeterminant form, such as the amoebae, rhizopods, and flagellates, as well as the bacteria. Most algae, including the blue-green algae, were not considered Protista at all, but plants. Only much later, when other workers had shown that Haeckel's ideas on the lack of nucleus in many protistans was wrong (19), did Haeckel place the simple blue-green algae (*Chroococcaceae*) within the group Monera.

Haeckel's views of biological systematics were derived primarily from his strong belief in Darwinism and his desire to develop a "natural" classification based on evolutionary descent. A passionate microscopist at a time before the advent of oil immersion lenses and aniline dyes, Haeckel's ideas were mostly wrong, but because of his prolific writing and polemical approach, his influence was widespread. He believed that the first organism to arise on earth (by spontaneous generation) was a type of moneran, a formless blob of protoplasm, devoid of nucleus (and also devoid of a membrane) and that such an organism was the forerunner of all subsequent life. As has been pointed out by others (47, 60), Haeckel's ideas were rarely based on his own observations, but he adapted the research of others to "fit" his preconceived ideas. Unfortunately, Haeckel's influence was so strong that it has been carried down in subsequent thinking. Even as late as 1957, an influential textbook of microbiology was still using Haeckel's classification of Protista (62), classifying the living world into three kingdoms, plants, animals, and protists. The protists themselves are divided by Stanier et al. (62) into two groups reminiscent of Haeckel, the higher protists (algae, protozoa, and fungi) and the lower protists (bacteria and blue-green algae).

Haeckel's influence has also continued to be felt in the general field of systematics. Haeckel's distinctions were

retained by H. F. Copeland in his four-kingdom classification, but here the bacteria (Monera) were separated out from the other protists (the latter were called Protoctista by Copeland) as a separate kingdom. Copeland's kingdom Monera was retained by Whittaker in his widely cited five-kingdom classification (65). Whittaker retained the Monera because of the vast amount of evidence that had accumulated since Haeckel that made the bacteria (and blue-green algae) appear even more different from other living organisms. Haeckel's classification of Monera appears, in retrospect, to have been reasonable, but hardly for the reasons that Haeckel used.

The classification of the microbial world into protista and monerans served microbiologists for many years, but in 1962, Stanier and van Niel (64) published an influential and widely cited paper that suggested quite a different classification. Following an earlier suggestion by Chatton (10), the living world was divided into two major categories, procaryote and eucaryote, based primarily on the nature of the nucleus. Although it is clear that Stanier and van Niel were concerned primarily with the systematic position of bacteria in the living world, the distinction between two broad cell types based on the nature of the nucleus was to have strong influence on subsequent thinking about systematics and evolution. Stanier and van Niel first used the terms *procaryote* and *eucaryote* in English in this 1962 paper, but the terms had been coined (in French) by Chatton some years before (10). Considering how widely these terms pervade current biological writing, it is interesting that the term *procaryote* (*prokaryote*) is not listed in the 1967 *Random House Unabridged Dictionary* or *Webster's Third Unabridged Dictionary*, although the term *eucaryote* (preferred spelling) is listed in the latter, with the definition, "having a visibly evident nucleus." The kingdom *Procaryotae* was defined by Murray (46). (In his discussion, Murray makes no mention of Haeckel, Copeland, and Whittaker, all of whom had earlier established kingdom names for the exactly equivalent organisms.) The Greek prefix *pro* means *before* and is used by Murray in the context of primordial. This usage implies that the bacterial nucleus was the forerunner of the eucaryotic nucleus, although there is no evidence for this idea (68). The kingdom *Procaryotae* as formed by Murray included two major groups, bacteria and blue-green algae. Since 1974, when Murray's article was published, the term *blue-green algae* has almost disappeared, being replaced by the term *cyanobacteria*. Because of this, the term *procaryote* has little utility and in contemporary usage procaryote is equivalent to the term *bacteria*. The definition of the kingdom *Procaryotae*, as given by Murray, includes the following words about the bacterial nucleus: ". . . single cells or simple associations of similar cells (0.2–10 μm in smallest dimension) forming a Kingdom defined by cellular, not organismal, properties. The nucleoplasm (genophore) is never separated from the cytoplasm by a unit-membrane system (nuclear membrane) and is not associated with a basic protein. Cell division is not accompanied by cyclical changes in the texture or staining properties of either nucleoplasm or cytoplasm; a microtubular (spindle) system is not formed." It should be noted that this is primarily a negative definition, specifying characteristics of eucaryotes which are not found in procaryotes.

In the 1962 paper in which they introduce the terms *procaryote* and *eucaryote*, Stanier and van Niel (64) emphasize the central importance of the nucleus in classification. However, they were still strongly influenced by the "Robinow cytological nucleus" and did not attempt to relate the bacterial nucleus to the accepted concepts of genome and nucleic acid organization in bacteria except only in extremely general terms. It is curious that although the very terms *procaryote* and *eucaryote* refer to the nucleus, most of the evidence that Stanier and van Niel bring to bear on the desirability of their new classification concerns other structural features of procaryotes, such as the respiratory and photosynthetic apparatus (lack of compartmentation into mitochondria and chloroplasts), cell wall chemistry, flagellar structure, etc. What of the nucleus itself? ". . . bacteria . . . have a cellular organization, designated as procaryotic, which does not occur elsewhere in the living world. The principal distinguishing features of the procaryotic cell are . . . absence of internal membranes which separate the resting nucleus from the cytoplasm . . . nuclear division by fission, not by mitosis, a character possibly related to the presence of a single structure which carries all the genetic information of the cell." The use of "division by fission" indicates that Stanier and van Niel had not really assimilated recent ideas about the disposition of DNA in the bacterial cell. Since a nucleus is not present in these organisms, it is probably better not to try to think about the nucleus at all, but to think only in terms of DNA and the genome. Thus, instead of thinking of nuclear division, one should think of DNA replication and partioning. In much of the general writing on this subject, there has been considerable confusion about the difference between genome division and genome partitioning, two quite different processes.

GENOMES OF MITOCHONDRIA AND CHLOROPLASTS

At about the time that the nature of the bacterial genome was being clarified, new ideas and new information on two important organelles of the eucaryotic cell, the mitochondrion and the chloroplast, were being presented. Genetic studies had indicated that these organelles had some genetic information that operated independently of the nucleus. Biochemical studies indicated that these organelles had their own protein-synthesizing machinery that was "bacterialike." Electron microscopic studies (54) showed that DNA fibrils were present within these organellar structures. The idea is now firmly established that these organelles arose during evolution as "symbiotic" associations of eucaryotic cells with procaryotes, and subsequent loss of function during evolution has led to the extremely modified and simplified genetic systems these organelles appear to be today. The eucaryotic cell can thus be viewed as a chimera, but one in which the bulk of the genetic information is present in the nucleus.

MOLECULAR PHYLOGENY

In ending this article, a few words should be said about the exciting and exceedingly fruitful studies on molecular phylogeny based on sequence analyses of ribosomal RNA molecules (68). The general outline of phylogeny derived from these studies is of three primary kingdoms, called eucaryotes, eubacteria, and archaebacteria. Although the eubacteria and archaebacteria are both procaryotes, in the sense defined above, from a molecular phylogeny viewpoint they are no more closely related than either group is with the eucaryotes. (It is relevant to point out that Woese uses the term "procaryote" as equivalent to "bacteria.") As Fox et al. have stated (16): "This tripartite division of extant life is incompatible with the conventionally accepted view in which living systems are divided into two basic phylogenetic

categories, procaryotes and eucaryotes. However, the eucaryotic cell is now recognized to be a genetic chimera, whose evolutionary origins we do not yet understand. It is no longer permissible to take these two categories—or their equivalents as others define them—as phylogenetically comparable, much less to see them as exclusive. . . . The biologist today is accustomed to viewing "eucaryote-procaryote" as some fundamental phylogenetic dichotomy. However, this cannot be so."

The key point here is that ideas about the nature of the bacterial nucleus have pervaded thinking about classification for over 100 years. Most of these ideas have been unfruitful, based as they have been on improper facts, blind prejudice, and unclear thinking. Molecular phylogeny, in the Woese sense, will either ultimately settle questions of phylogeny and classification or open up a whole new set of questions and make the current ones moot.

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