NUCLEI AND LARGE BODIES OF LUMINOUS BACTERIA IN RELATION TO SALT CONCENTRATION, OSMOTIC PRESSURE, TEMPERATURE, AND URETHANE[1]

FRANK H. JOHNSON AND DAVID H. GRAY

Microbiological Laboratory, Princeton University, Princeton, New Jersey

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A number of investigators in recent years (cf. Robinow, 1945) have convincingly demonstrated the general, consistent occurrence in bacteria of discrete, Feulgen-positive units of chromatin, which in their various aspects have been referred to as chromatinic bodies, chromosomes, nucleoids, or nuclei. They may be seen in unstained cells, also, and have been recently identified in electron micrographs (Hillier, Mudd, and Smith, 1949). Much remains to be learned, however, concerning the fine structure of these bodies, their variation in gross form under normal conditions as well as under the influence of environmental factors, and their genetic significance, which is still purely a matter of inference.

The present study with marine luminous bacteria was undertaken with particular reference to the influence of salt concentration and osmotic pressure. These organisms are unusually favorable for the purpose in view inasmuch as comprehensive physiological, physical, immunological, and electron microscopical studies have been made in connection with the same factors (Harvey, 1940; Johnson, Zworykin, and Warren, 1943; Warren, 1945; Johnson, 1947). Optimal growth, luminescence, and respiration occur in sea water or isotonic (3 per cent) NaCl media, but if the cells are placed in greatly diluted salt solution (less than 0.3 per cent NaCl), respiration, viability, and growth cease; specific antigenic and surface-active substances are liberated into the medium; surface properties are affected; and "ghosts" remain in place of the normal cells. In isotonic (0.73 molal, or roughly 25 per cent) sucrose such changes do not occur at once, showing that the drastic effects are primarily osmotic. In concentrated sea water the cells shrink and their physiological activity and surface properties are again affected.

The physiological effects of salt concentration, of temperatures above the normal optimum, and of drugs such as urethane are largely reversible within certain limits of concentration and temperatures. Reversible changes also occur, under certain conditions, in the appearance of the nuclei, as shown by the present investigation. In addition, the hitherto unreported occurrence of "large bodies" in young cultures of these bacteria has been found.

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METHODS

Although species of both rod-shaped (*Achromobacter fischeri*) and coccoid cells (*Photobacterium splendidum*)[2] were studied, most of the work was done with the former, which provided somewhat more favorable material. Both species were routinely maintained on nutrient agar containing 3 per cent NaCl, 1 per cent glycerol, and about 0.5 per cent $CaCO_3$. For the nuclear stains, two successive plates of brain-heart agar plus 3 per cent NaCl were always prepared. The first was inoculated with a suspension of cells from a 1-day-old slant of maintenance medium. With *A. fischeri* this plate was incubated for 4 hours at the optimum temperature of 28 C, and the young growth was used to inoculate the second plate. The latter was incubated from 1.5 to 6 hours, as described later, usually at 28 C. Cultures of the psychrophilic species, *Photobacterium phosphoreum*, were incubated at 15 C.

The staining methods of Robinow, involving osmic fixation of cells on the agar surface, hydrolysis of impression films in N HCl at 55 C, and staining in dilute, phosphate-buffered Giemsa blood stain, were followed throughout.

The effects of tonicity and salts were studied in two ways. For the first method, permitting gradual changes, square holes, about 1.5 inches on a side, were cut in a sheet of cork, $\frac{1}{4}$-inch thick. Several strands of thin chromel wire were stretched across the under surface of the cork at each hole. A series of small agar blocks from young plate cultures could thus be mounted with their lower surfaces in contact with a solution of the desired concentration of salt or other constituents, to allow diffusion of the solutes between the agar block and the other solution. A large volume of the latter in an enamel pan was always employed. When a block from a luminescing culture on 3 per cent NaCl agar was thus placed against distilled water at room temperature, the brightness of the light gradually dimmed over a period of about 45 minutes and disappeared completely in slightly more than 1 hour. Calculations based on the rate of diffusion of NaCl through an agar block 2 mm in thickness indicated that most of the salt is removed within about an hour.

The reversibility of the effects observed after dialyzing against a given solution was tested by placing some similarly treated, but unfixed, agar blocks back on 3 per cent NaCl solution and allowing at least 1 hour for diffusion. The cells were then fixed and stained for nuclei by the standard procedure.

A second method, for the sudden reduction of salt concentration, was adding 1 ml of a 3 per cent NaCl suspension of cells from a young plate culture to 19 ml of distilled water, thus giving a final salt content of about 0.15 per cent, which results in almost instantaneous cytolysis. A portion of the diluted suspension was then spread on the surface of 2 per cent plain, nonnutrient agar containing no added salt, and agar blocks were fixed in osmic acid vapor in the usual manner. For rapid increases in salt concentration, a weighed amount of salt was added to a suspension of cells in isotonic solution. A portion was then

[2] This culture, with the nomenclature used, was provided in 1939, through the courtesy of Professor A. J. Kluyver of the Laboratorium voor Microbiologie of the Technische Hoogeschool at Delft.

similarly spread on nonnutrient agar of a corresponding salt content, followed by osmic fixation.

Photomicrographs were taken with monochromatic light, using a Wratten filter no. 77 X to isolate the 546 mμ line in the emission spectrum of an AH-4 high-pressure mercury vapor lamp. A 95 × achromatic objective and 12 × ocular were used throughout.

RESULTS

Normal structure. Typical nuclear stains of coccoid and of rod-shaped cells of *Photobacterium phosphoreum*[2] and *Achromobacter fischeri*, respectively, each from young cultures at optimum temperatures, are shown in figures 1 to 4. In the former species (figure 1) the nuclear material appears variously as a darkly staining central body, or in two or more units distributed in a manner suggestive of changes similar to those accompanying mitosis in higher organisms. States resembling the anaphase are especially prominent, but examples are also evident which in appearance resemble the other phases of mitosis, including metaphase as seen in a polar view.

In *A. fischeri*, the chromatin of these young cultures appears mostly in the form of small granules, resembling the condition in older (18-hour) cultures of ordinary rods. The granules range in size from about 0.6 microns down to the limits of resolution of the microscope. The average size of distinct, apparently separate particles is greater than that of very small chromosomes, measuring 0.1 by 0.18 microns, in certain flowering plants (Blackburn, 1932–1933). The dumbbell-, X-, Y-, and V-shaped bodies, characteristic of the nuclei in young rods of common species (Robinow, 1945) are not so readily apparent in these preparations, although they can be detected by careful study (cf. figures 2a, 3a, and 4a). Moreover, such forms become conspicuous under the influence of increased salt concentration as described presently. Cultures of 1.5 to 2 hours' incubation at 28 C have long, snake forms (figure 2), which in the course of 3 or 4 more hours of incubation are largely replaced by numerous short and smaller cells (figure 3). The younger cultures frequently contain long filaments apparently devoid of nuclear material and some filaments in which the chromatin occurs as a continuous strand rather than as small discrete units.

The strain of *A. fischeri* used in this study dissociates, on maintenance medium, as dark colonies that are otherwise indistinguishable, through ordinary means, from those that emit light. Dark strains obtained from such colonies were examined, by the same procedure used for the luminescent cultures, for possible differences in appearance of the nuclei, but no differences were detected. Preliminary experiments were undertaken, from a genetic point of view, to determine whether luminescence would reappear in mixed cultures of dark variants, but the results have not been conclusive.

Large bodies of A. fischeri. Direct microscopic observations of living cultures showed that although "large bodies" were not present immediately after inoculation of a new plate from a 4-hour culture, they soon appeared, sometimes in considerable abundance (figure 4). They were produced from apparently normal

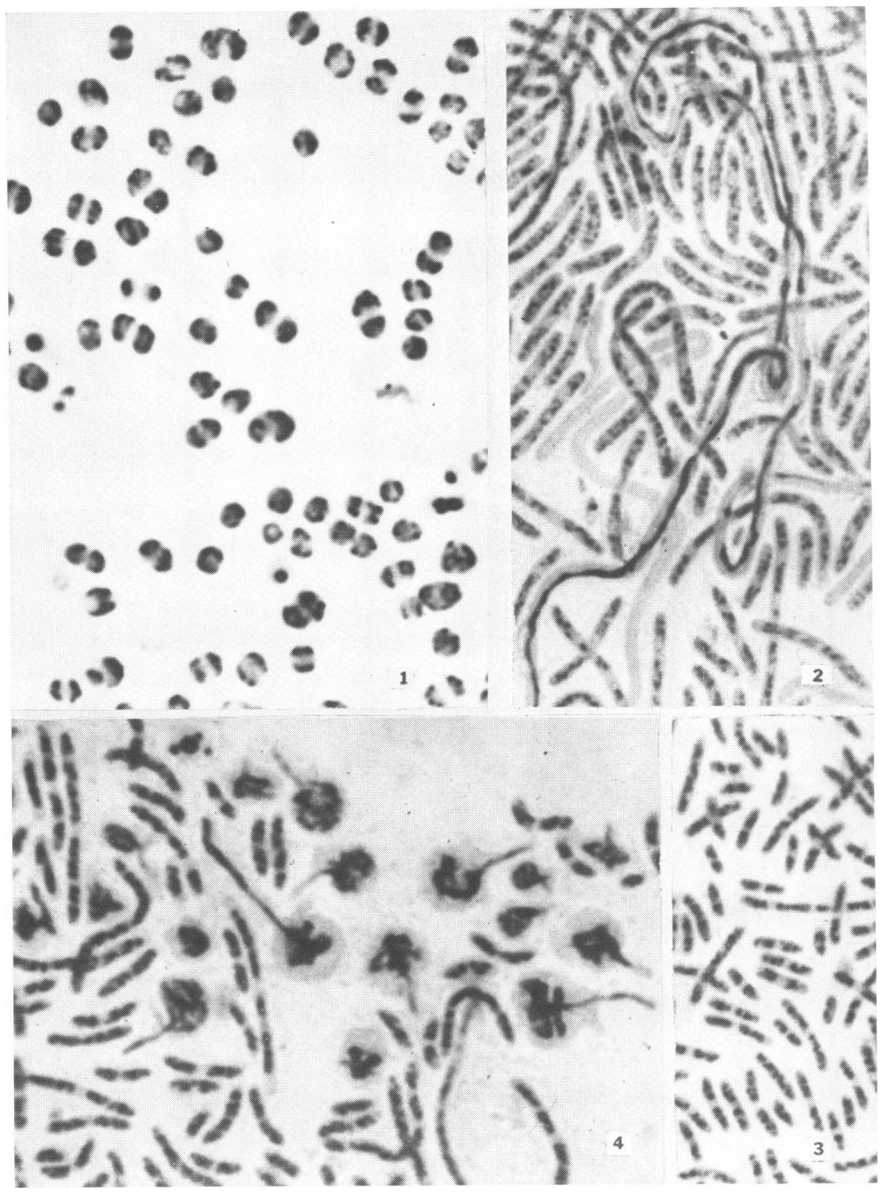
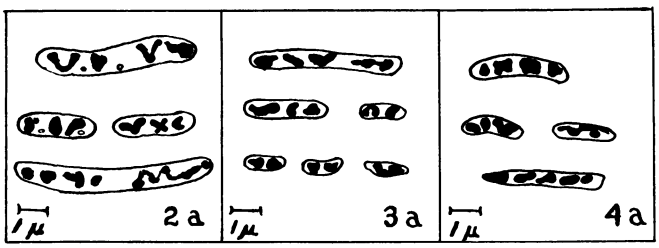


Figure 1. Young cells of *P. phosphoreum;* × 2,400.
Figure 2. A. fischeri, 1¾-hour culture; × 2,400.
Figure 3. A. fischeri, 6-hour culture; × 2,400.
Figure 4. A. fischeri, 4-hour culture, showing large bodies; × 2,800.

cells by a terminal or lateral swelling, which gradually enlarged and underwent changes in shape, in the manner illustrated by the diagrams in figure 5, resembling a similar phenomenon in cultures of *Proteus* (Hutchinson and Stempen, 1949). In the later stages, small granules were apparent within these bodies or at the

surface, seemingly in part on the outside. Although we have not succeeded in determining the fate of such bodies, which generally become overgrown by the more rapidly developing small cells, the granulated appearance suggests that they may ultimately give rise to the more typical cells, as in *Bacteroides* (Dienes and Smith, 1944) and in *Proteus* (Hutchinson and Stempen, 1949).



Figures 2a, 3a, and 4a. Diagrams, approximately to the scale indicated, of some of the cells in figures 2, 3, and 4, respectively.

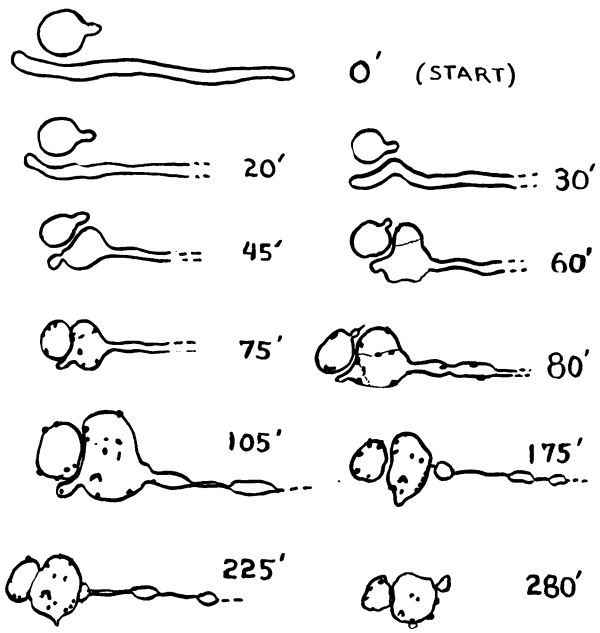
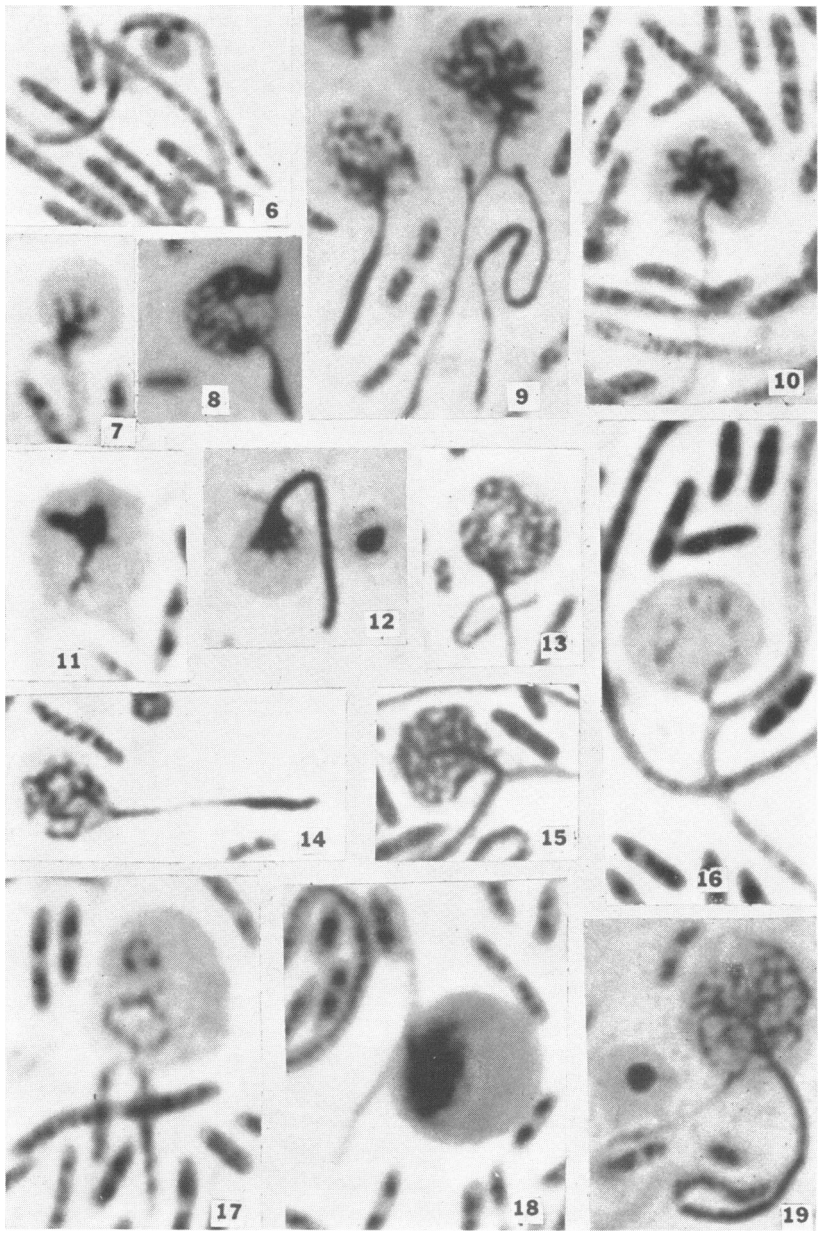


Figure 5. Diagram of formation of large bodies as observed in growing cultures of *A. fischeri*.

It was not unusual for two large bodies to form near each other on adjacent cells and to come into contact as they developed, as indicated in figure 5. There was no certain evidence that fusion ever occurred, although this possibility cannot be excluded on the basis of the observations that were made.

Among a large number of stained preparations exhibiting bodies with different appearances of the nuclear material, there were always some individuals that



Figures 6 to 19. Representative large bodies in young cultures of *A. fischeri;* × 3,200. Figures 7, 11, and 16 to 18 from 5-hour cultures at 15 C; the others from 2- to 4-hour cultures at 28 C.

showed a general correspondence in their patterns. Representative examples are illustrated in figures 6 to 19.

Influence of hypotonicity. When agar blocks from a young culture are dialyzed against distilled water according to the method described, the nuclear material

gradually loses its stainability and discrete form as the solutes gradually diffuse out of the agar. An intermediate stage is illustrated in figure 20. The end result of prolonged dialysis is practically complete cytolysis (figures 22 to 24), in which the cells show only a vague or sometimes no clear indication of their former nuclear bodies. Moreover, they generally appear larger, as if by collapse accompanying loss of contents, somewhat like cells acted upon by lysozyme (Welshimer and Robinow, 1949, figures 3 and 4). The rate and extent of these changes were apparently greatest in the youngest cells (1- to 2-hour cultures), but the sequence and general picture were always the same.

At an intermediate stage of reduced salt concentration (e.g., as represented in figure 20) recovery of essentially the normal nuclear appearance takes place if the agar block is placed for an equivalent period of time in contact with 3 per cent NaCl solution (figure 25; compare with figure 2). This remarkable reversibility has a parallel in physiological activity in that both the reduced respiratory rate and the decreased luminescence intensity that occur in sea water diluted to 18 per cent of its normal concentration, by the addition of distilled water, undergo a large measure of recovery when concentrated sea water is added to restore the previous salt concentration (Johnson and Harvey, 1938). It is clear, therefore, that neither the functional activity of the cell nor the basic structure of the nucleus is destroyed by such treatment.

The preparation illustrated in figure 25 showed a somewhat unusual abundance of "X-bodies," i.e., long cells with a single, heavily staining, elongated central mass of chromatin, clear at each end, resembling those that have been observed by Robinow in other species of bacteria. Moreover, there appear to be intermediate stages toward the formation of normal cells from X-bodies, or vice versa (figures 26 to 32).

No reversibility of nuclear changes occurs after complete cytolysis resulting from either gradual or sudden reduction of salt beyond a critical concentration.

Distinction between hypotonicity and dilution of salts. When placed against isotonic sucrose, a temporary decrease in osmotic pressure in an agar block occurs because of the difference in rates of diffusion of NaCl and of sucrose. According to calculations, the minimum is reached at about 35 minutes, with the reduction amounting to 49 per cent. After 1 hour the initial osmotic pressure is largely restored, but the nuclear material of cells in the isotonic solution of the nonelectrolyte assumes an appearance similar in certain respects to that characteristic of incomplete cytolysis (figure 21). This change is largely reversible, as shown by subsequently placing similar blocks against 3 per cent NaCl for 1 hour. Thus, although osmotic pressure is apparently a contributing factor, the presence of electrolytes is obviously important to the normal state of the nucleus. The mechanism of these reversible changes in the nucleus is not clear, particularly because the plasma membrane is generally assumed to be practically impermeable to salt. The irreversible changes of complete cytolysis evidently result from the rupture of the cell, with irreparable disorganization of its structure, when the osmotic pressure gradient across the cell surface exceeds the mechanical strength of the wall.

Influence of concentrated salt solution. A gradual increase in salt concentration

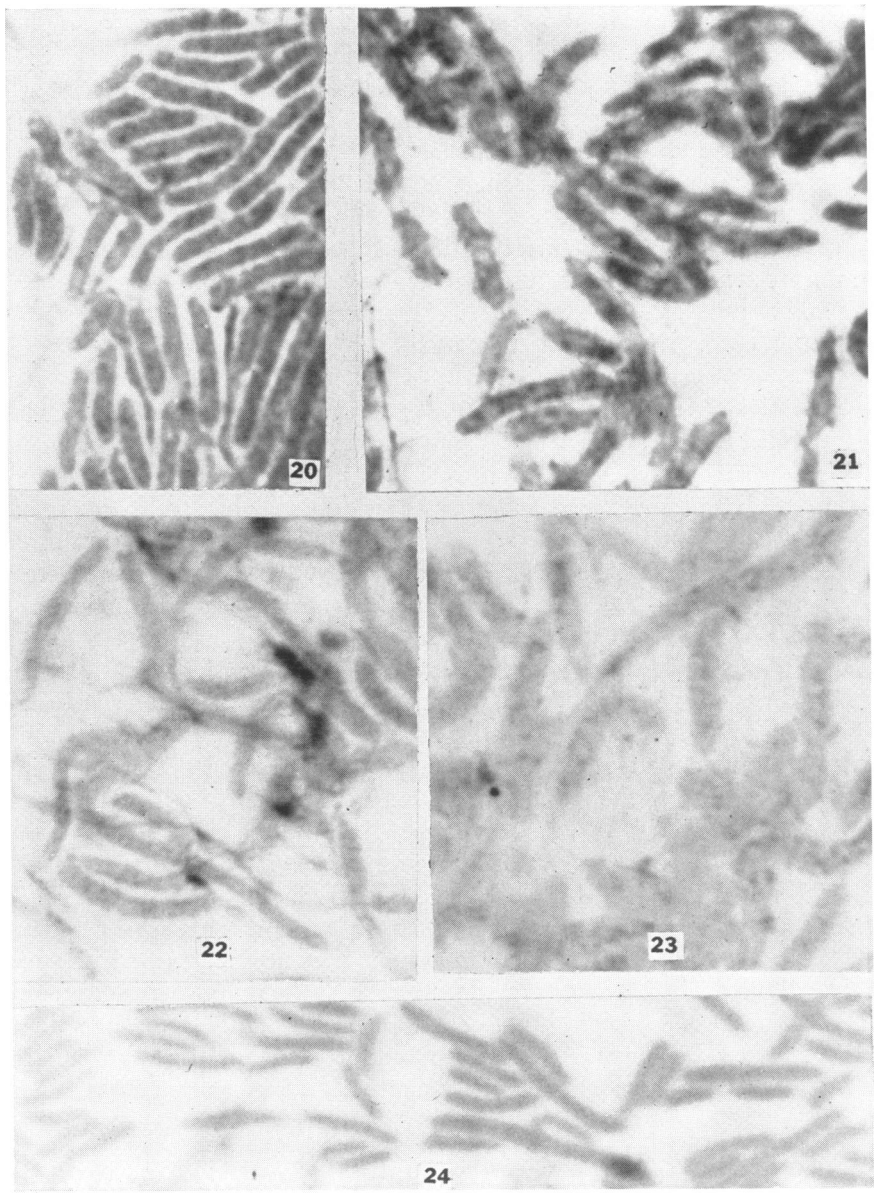


Figure 20. Incomplete cytolysis of *A. fischeri* in dilute NaCl; × 3,300.
Figure 21. A. fischeri in isotonic sucrose; × 3,100.
Figures 22 and 23. Late cytolysis of *A. fischeri* in dilute NaCl; × 3,400.
Figure 24. A. fischeri from 6-hour culture, after removal of most of the NaCl, leaving no distinct nuclear structure; × 3,400.

to 20 per cent NaCl causes the chromatin to stain more intensively and apparently to coalesce into fewer but larger units with the dumbbell, V, and other shapes typical of those in young cultures of common species of rods (figures

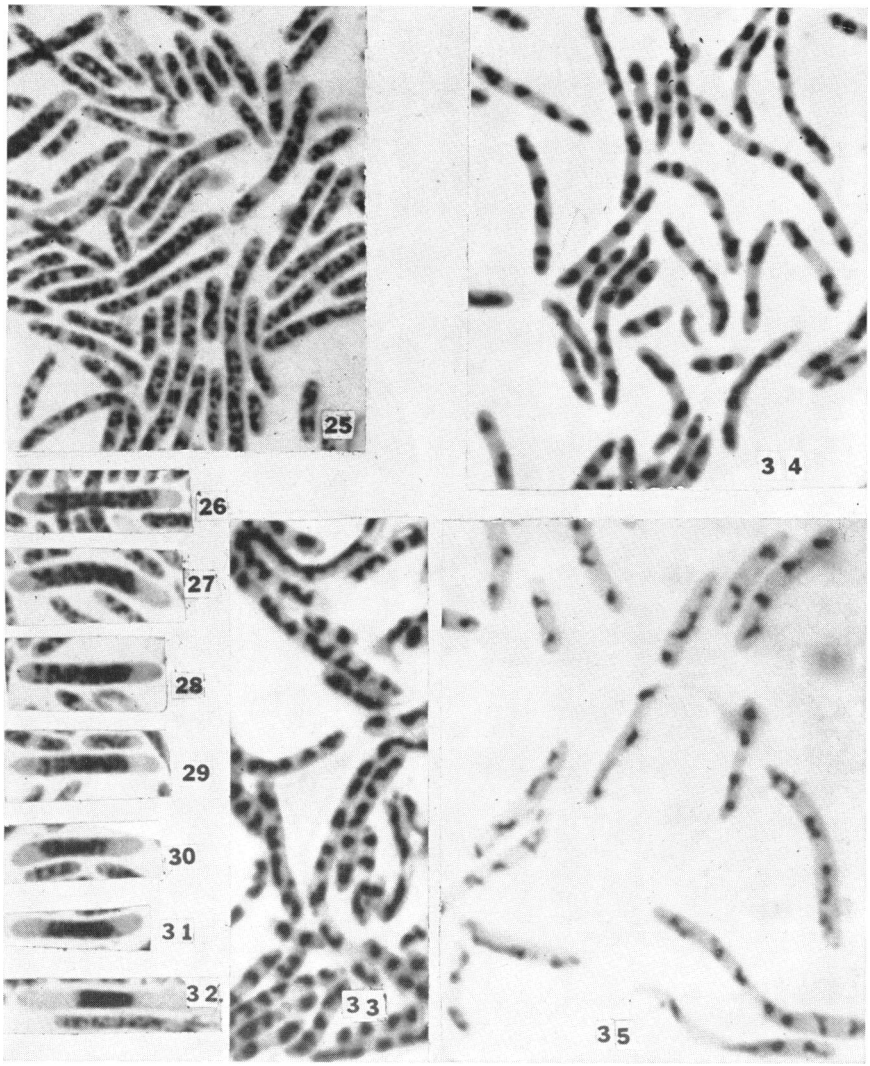


Figure 25. "Reversed cytolysis" of *A. fischeri;* × 2,700.

Figures 26 to 32. Possible stages in the formation of an "X-body" from normal cells, or vice versa; × 2,700.

Figure 33. Effects of gradually increased NaCl to 20 per cent on nuclear structure of *A. fischeri*, 2.5-hour culture; × 3,400.

Figures 34 and 35. Similar to figure 33, but with cells from a 4-hour culture, and counterstaining with fuchsin; × 3,400.

33 to 35). According to Robinow, older cultures (10 hours at 37 C) of the latter have more dispersed chromatin, which appears to coalesce when the cells are transferred to fresh medium, thus resembling the change in young cultures of *A. fischeri* under the influence of concentrated NaCl. The salt effect is reversible, but there was some indication that, after long contact with 20 per cent NaCl,

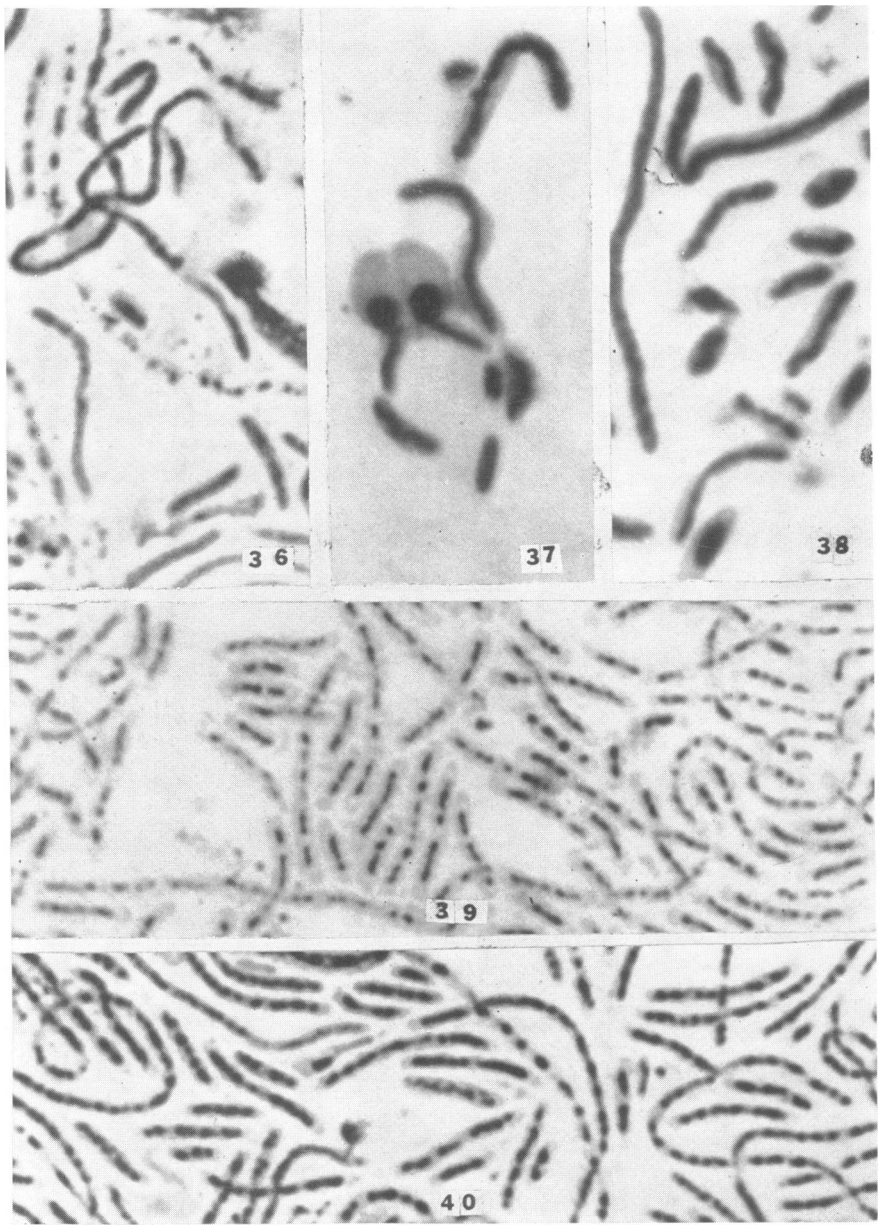


Figure 36. A. fischeri, after incubating for 80 minutes at 37 C.

Figures 37 and 38. Effects of sudden increase in NaCl to 20 per cent on nuclear structure of *A. fischeri;* × 3,100. The large body in figure 37 has an appearance suggestive of fusion or fission.

Figures 39 and 40. Cells from a 4-hour culture of *A. fischeri* after exposure to 0.5 M urethane for 35 minutes (figure 40) and for 63 minutes (figure 39), counterstained with fuchsin; × 3,100.

when the agar block was subsequently placed against 3 per cent NaCl, the cells underwent swelling, followed by changes resembling those that occur in a hypotonic medium.

Sudden increase in salt concentration, by direct addition to a suspension of cells, caused the chromatin to form an apparently continuous, wavy strand extending the length of the cell (figures 37 and 38). This change could not be reversed by the gradual removal of the salt.

Influence of above-optimal temperatures. At temperatures higher than 30 C, *A. fischeri* does not produce appreciable growth. When incubated for only 80 minutes at 37 C, the normal nuclear pattern of young cells appears disrupted, and the chromatin assumes the form of either a continuous or a slightly beaded thread (figure 36) somewhat like that resulting from a sudden increase in salt concentration. During subsequent incubation of such plates at room temperature, however, normal growth and luminescence took place, showing that not all the cells were killed by the fairly short exposure to 37 C. After incubation for several hours at this temperature, however, the nuclear structure varied from practically complete disintegration, only a few granules remaining, to strings of small, chromatinic spheres.

Influence of urethane. Depending upon concentration and temperature, urethane and related drugs catalyze a reversible and an irreversible denaturation of certain proteins both in living cells and in extracted systems. In effect, urethane lowers the temperature for thermal denaturation. In a concentration of 0.1 M, which causes a reversible inhibition of luminescence, there was no apparent change in the nuclei. At 0.2 M, which causes a greater inhibition of luminescence and a slow destruction of the system at room temperature, some pycnosis of the nuclear material could be detected. Higher concentrations of 0.4 to 0.5 M urethane, leading to irreversible physiological effects and preventing growth, also caused irreversible changes in the nuclei. The normally granular chromatinic structure was replaced even within a short period of time by a fairly dense, more or less continuous strand, or a line of small, apparently individual spheres (figures 39 and 40). These concentrations are of the same magnitude as those which Burt (1945) found prevented mitosis, caused pycnosis, and tended to revert the actively dividing nucleus of *Colpoda steinii* to the resting stage.

Significance of the effects of salt concentration, heat, and urethane. The changes in the nuclear material of *A. fischeri*, under the influence of the factors considered, are summarized schematically in figure 41. The double arrows indicate changes that are apparently easily reversible, and the single arrows those that are not. It is reasonable to believe that the latter type involves a denaturation of nucleoproteins by above-optimum temperatures, 0.5 M urethane at room temperature, or the sudden increase in NaCl concentration to 20 per cent.

In connection with this interpretation, the observations of Klieneberger-Nobel (1945) concerning various anaerobic species are of particular interest. Within a few minutes after exposure of the cells to air, the nuclear material begins to fuse and to take on an appearance resembling that in luminous bacteria after treatment with heat, urethane, or sudden increase in salt concentration (cf. her figures

6, 8, 10, 11, and 12). The well-known inimical effects of oxygen on obligate anaerobes makes it reasonable to conclude that the mechanism of the nuclear changes in both cases involves protein denaturation.

The irreversible effects of greatly diluted salt solution, on the basis of various lines of evidence already referred to, involve the osmotic rupture of the cell, with loss of some of its contents and general disorganization of its structure.

The reversible changes under the influence of salt concentration and electrolytes show that the nucleus of a given bacterial cell has a remarkable capacity to

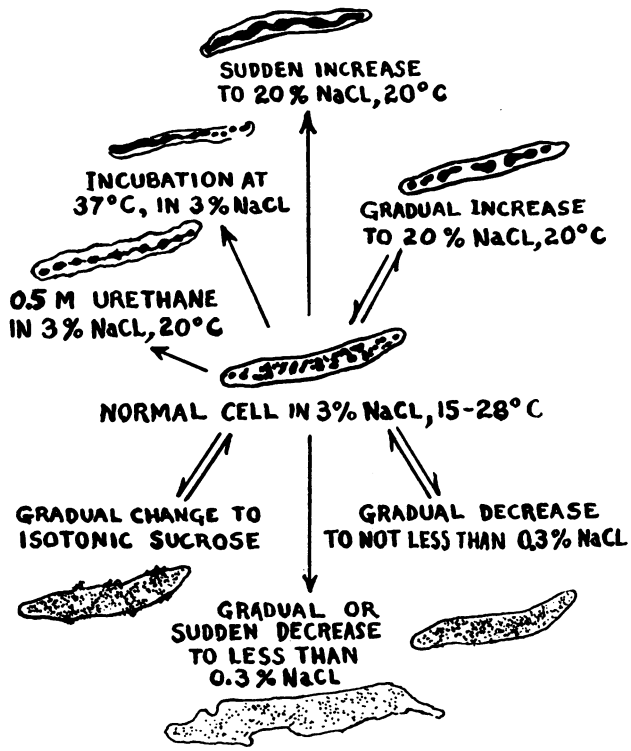


Figure 41. Diagrammatic representation of the changes in the appearance of the chromatin of *A. fischeri* under the various influences discussed in the text.

assume diverse appearances, varying from typical, densely staining Robinow bodies to seemingly small, dispersed, and lightly staining granules, without destruction of its basic organization. The differences in appearances are, however, no more nor less remarkable than those accompanying normal mitosis and differentiation in cells of higher organisms. Thus, some of the variations in nuclear structure that have been observed in other types of bacteria and within a single strain under different conditions, such as age of culture, are not surprising. They perhaps reflect merely the processes of normal cell division in bacteria and the influence of chemical differences in the internal cellular environment associated with different stages of division, physiological states, or conditions of the medium. Although a complete definition of the bacterial nucleus must await the

accumulation of further evidence concerning its structure, organization, and function, it would obviously be a mistake to apply the term "nucleus" to any particular one of the diverse forms into which it reversibly enters.

Although the exact nature of the chromatin granules and their relation to individual nuclei in bacteria remain to be established, the influence of electrolytes, i.e., salt vs. sucrose, has a parallel in the influence of the same factors on the interphase nucleus of both plants and animals. Ris and Mirsky (1949) have shown that in nonelectrolyte solutions (sucrose, glycerol) the isolated nuclei stain lightly and homogeneously by the Feulgen method or by methyl green, specific for desoxyribonucleic acid (DNA). In physiological salt solution the chromatin structure becomes visible and the staining is more intense and confined to the visible structure. The effects are reversible on alternately placing the nuclei in sucrose and physiological salt solutions. Moreover, these changes are fully accounted for by changes in the state of the chromosomes, through a reversible extension and condensation of the DNA component. The behavior of animal and plant cell nuclei in strong salt solution is more complex, and is apparently not directly comparable to that of the nuclear material in the halophilic, marine luminous bacteria, but the similarities in the effects of electrolytes concern such a fundamental property of chromosomal material that it can scarcely be without significance. On this as well as other grounds, it seems very probable that there exists in bacterial cells a true homologue to the nuclei in plant and animal cells generally.

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SUMMARY

The nuclei in marine luminous bacteria, *Photobacterium phosphoreum* (coccoid cells) and *Achromobacter fischeri* (rods), were studied by the staining methods of Robinow. In young cells of the former species on isotonic 3 per cent NaCl, brain-heart agar, the chromatin is distributed in a manner resembling various stages of mitotic cell division. In the latter species, young cultures consist mostly of long rods containing numerous small units of chromatin, but on the average larger than the smallest known chromosomes of higher organisms, varying in shape from spheroid to dumbbell, V, Y, and other shapes.

Large bodies occur abundantly in young cultures of *A. fischeri* on 3 per cent NaCl, brain-heart agar at optimum temperature. They form by lateral or terminal outpocketings from normal cells, but their ultimate fate was not determined. In stained preparations, the chromatin in large bodies has various appearances, but among numerous specimens some generally similar patterns are evident.

Dialysis of the salt from thin agar blocks of *A. fischeri* leads to cytolysis of the

cells, in the final stages accompanied by complete disintegration of the nuclear structure and rupture of the cells. Moderate reduction of the salt concentration, causing incomplete cytolysis, leads to a decrease in stainability of the chromatin, which also appears more finely dispersed, but this change can be reversed by dialyzing again against isotonic (3 per cent) NaCl. Dialysis against isotonic (0.73) molal sucrose causes reversible changes in appearance of the nucleus resembling those of incomplete cytolysis.

With *A. fischeri*, gradual increase in salt concentration of the medium to 20 per cent NaCl, by diffusion into thin agar blocks, causes the chromatin apparently to coalesce or aggregate into larger units, similar in appearance to the bodies found in young rods of ordinary bacterial species. This change is reversible on dialysis again against isotonic NaCl. Sudden increase in salt concentration, by the addition of an amount to make it 20 per cent in a suspension of cells, causes an irreversible change, the chromatin assuming the form of an apparently continuous thread extending throughout the length of the cell.

On brief incubation of *A. fischeri* at 37 C, about 10 degrees above the normal optimum, the chromatin tends to coalesce in the form of long, apparently continuous or sometimes beaded threads. At a much lower temperature, about 20 C, 0.5 M urethane causes an irreversible pycnosis of the chromatin into threads of spheroids resembling those that form under the influence of heat alone.

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