

PLOIDY STUDIES ON THE LARGE CELLS OF *MICROCOCCUS AUREUS*¹

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The determination of ploidy in bacterial cells has been unsatisfactory in most cases because the criteria of ploidy in higher forms can not as yet be applied directly to bacteria. The best evidence of diploidy in bacteria has been in the various "Het" strains of *Escherichia coli* strain K-12 developed by Lederberg *et al.* (1951). Unfortunately, genetic recombination has not been found in many bacteria, so this means of ploidy determination has been very limited in use. Genetic and radiobiological studies indicate that bacteria are usually haploid (Lea, 1947); however, several instances have been reported in which cytological information suggests a diploid phase (Bisset, 1950).

Lindgren (1942) presented cytological evidence for a meiotic division in *Micrococcus orchraceous*. Webb and Clark (1954) found evidence of a diploid or polyploid phase in a life cycle of *M. aureus*. These large cells appeared cytologically to undergo a meiotic division to yield normal haploid cells. This paper is a continuation of the studies of the large, apparently diploid cells of *M. aureus*.

MATERIALS AND METHODS

The organisms used in this study were *M. pyogenes* var. *aureus* strain FDA 209, nine other strains of *M. aureus*, and thirteen other cocci, all obtained from the University of Oklahoma Culture Collection. These organisms were maintained in stock on nutrient agar (Difco), and all experiments were performed on nutrient agar unless otherwise noted.

Radiation experiments were performed using a General Electric low-pressure mercury vapor ultraviolet light and a Picker Army Field X-ray of 30 ma output. In the ultraviolet studies ir-

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radiation was done at a distance of 14 inches from a cell suspension in 0.85 per cent saline or from the agar of a petri dish which had been surface plated with approximately 300 viable cells. In X-ray experiments cell suspensions in 0.85 per cent saline were irradiated with the tube operating at 75 kv and 16 ma at a target distance of 5 inches.

In order to eliminate misleading results due to clumping, cell suspensions were shaken vigorously for 5 to 10 minutes with glass beads in a screw cap test tube. This procedure yielded suspensions of 95 per cent single cells as revealed by the phase microscope. Cross septation was further checked in all suspensions by using a cell wall stain (Webb, 1954).

Nuclear studies were performed using the crystal violet nuclear stain (Chance, 1952; Clark and Webb, 1955a) the acid giemsa stain (Murray *et al.*, 1950) and the thionin-SO₂ technic (DeLamater, 1951). Phase studies were performed with American Optical dark contrast medium and B-minus contrast medium objectives.

RESULTS

Conditions of formation. In order to adequately investigate the large cells of *M. aureus* it was necessary to determine the conditions of culture which caused the maximum formation of these cells, since cultures containing at least 75 per cent large cells were desired. Under the usual cultural conditions the occurrence of large cells was erratic. They were found to appear at a maximum incidence after 8 hours of growth and to disappear rapidly after 12 hours of incubation (table 1). It was also found that the presence of excess moisture on the nutrient agar surface caused an increased incidence. Few, if any, large cells would form on a dried agar surface. This was not directly associated with medium age since old, partially dried agar would yield a high percentage of the cells after it had been melted and resolidified to yield a moist surface.

The presence of carbohydrates interfered with large cell formation. Only a few such cells were observed when any carbohydrate was incorporated in the nutrient agar. The stage of growth of the inoculum was also significant in that the maximum yield of large cells was noted when resting cells were used.

Another significant condition for large cell formation was the temperature of incubation. As shown in table 1, the maximum yield of such cells occurred when the cultures were incubated at 25 C. By fulfilling conditions of temperature, moisture, inoculum age, and time, it was possible to consistently produce cultures containing 70 to 90 per cent large cells, but this percentage diminished rapidly as the culture aged.

It was also found that there was a correlation between the incubation temperature and the degree of clumping. A high incidence of clumped cells was found in cultures incubated at 37 C, and the proportion of clumps decreased as incubation temperature was decreased.

Incidence of formation. Nine strains of *M. aureus* were examined to see if the formation of large cells was a restricted phenomenon. All nine strains were found to produce these cells with varying incidence under similar cultural conditions. The FDA 209 strain yielded the highest percentage of large cells under the conditions tested, but it appeared probable that the conditions for maximum formation varied from strain to strain. A similar large cell formation was also observed in other cocci (table 2). These cultures were observed at both 9 hours and 7 days, and if large cells were observed the culture was recorded as positive. Seven of the 13 various cocci

TABLE 1

Effect of time of incubation on the occurrence of large cells in Micrococcus aureus

Time of Incubation	Percentage Large Cells at Incubation Temperatures of			
	20 C	25 C	30 C	37 C
hr				
0	0	0	0	0
5	38	50	1	1
8	65	80	1	1
10	65	75	1	1
12	50	69	1	1
14	36	55	1	1
24	36	1	0	0

TABLE 2

The occurrence of large cells in various cocci

Organism	Large Cells Present	
	9-hr culture	7-day culture
<i>Micrococcus flava</i>	—	—
<i>M. perflava</i>	—	+
<i>M. candidus</i>	—	—
<i>M. ureae</i>	+	—
<i>M. citreus</i>	—	—
<i>M. pyogenes</i> var. <i>albus</i>	+	±
Unknown hemolytic micrococcus	+	+
<i>Streptococcus lactis</i>	+	+
<i>S. pyogenes</i>	—	—
<i>Diplococcus pneumoniae</i>	+	+
<i>Sarcina lutea</i>	—	—
<i>Sarcina flava</i>	+	+
<i>Gaffkya tetragena</i>	+	—

cultures tested were found to contain large cells after 9 hours' incubation. In the case of *M. perflava*, large cells were found only in old cultures.

Mechanism of formation. Repeated studies were made on living cultures of *M. aureus* using the phase microscope in an effort to determine the origin of the large cells. In no case was any cellular fusion seen, and in several instances isolated normal cells were observed to increase in size and divide as large cells. Although no nuclear activity was observable in those cases with the phase microscope, this is suggestive of an autogamy or endopolyploidy. An intracellular nuclear fusion would cause a ploidy increase, and there is some cytological evidence for such in bacteria (Bisset, 1950).

The large cells were studied cytologically using the crystal violet nuclear stain (Chance, 1952), the acid giemsa stain (Murray *et al.*, 1950), the thionin-SO₂ stains (DeLamater, 1951), and a cell wall stain (Webb, 1954).

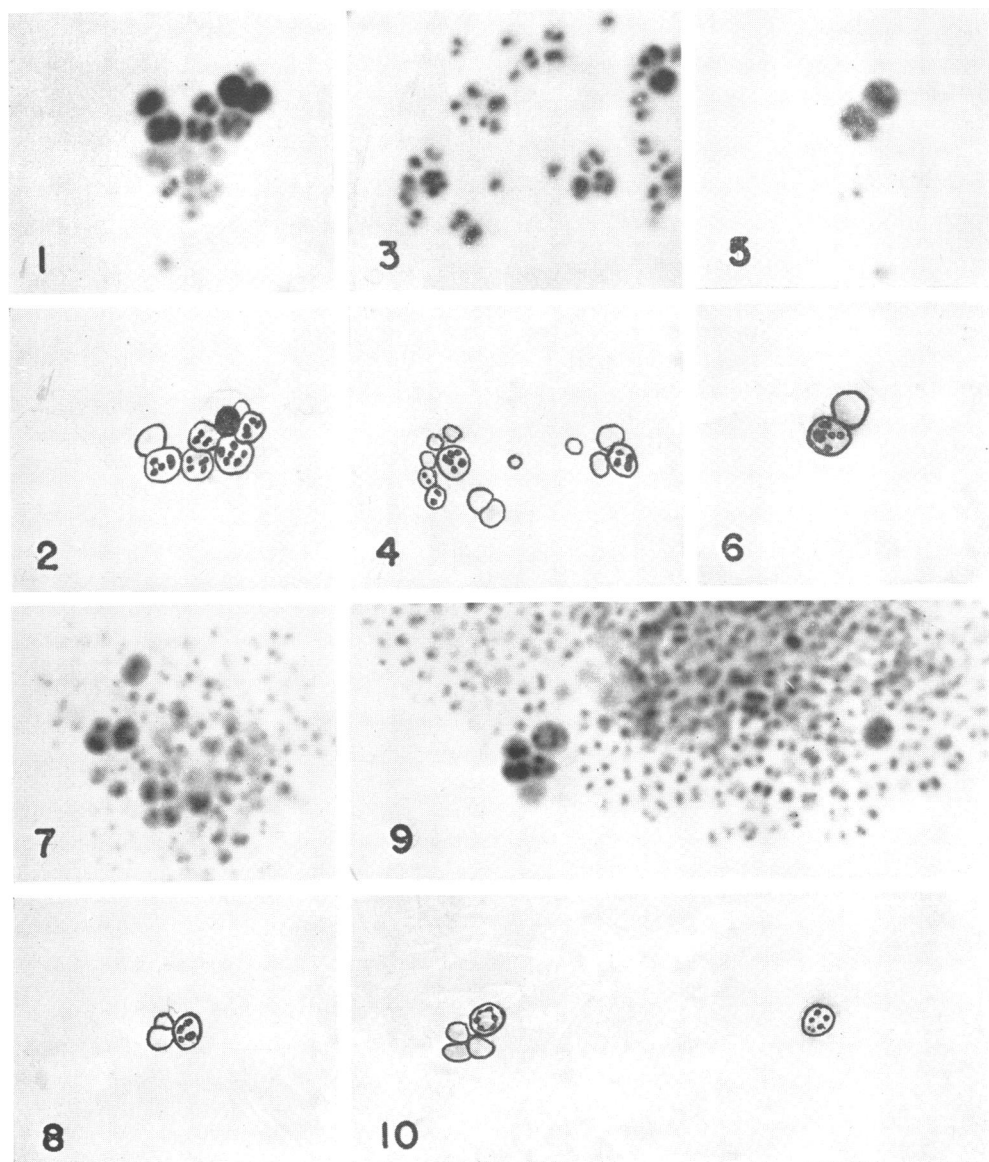
In the thionin-CO₂ staining procedure the freeze-dehydration process was omitted and the preparations were examined and photographed as temporary water mounts (DeLamater, 1951). It should be recognized that the freeze-dehydration procedure is not an essential part of the staining reaction but rather functions to yield permanent slides with perhaps a slight increase in nuclear definition.

Large cells were produced by streaking an

inoculum of a 4-day-old culture on the surface of a petri plate and incubating the plate at 25 C under semihumid conditions for 6 to 8 hours. Under these conditions only a small portion of

the cells were found to be the large type, but the incidence was sufficient for cytological studies.

The acid giemsa stain yielded results which appeared very similar to that of the crystal violet



Figures 1-10

Figures 1, 3, 5, 7, and 9 are photographs of large cells stained with the thionin-SO₂ method and showing 4 or 6 nuclear components.

Figures 2, 4, 6, 8 and 10 are drawings of the significant cells in the photographs to better reveal the appearance of the nuclear structure.

Note in figures 2 and 3 the normal *Micrococcus aureus* cells with two internal structures in non-septate cells. All photographs magnified 3250X.

nuclear stain (Webb and Clark, 1954) except that no cell plates were stained. The nucleus appeared elliptical in resting cells and became round prior to division. Nuclei of the large cells were approximately twice the diameter of small cell nuclei. The nuclei were less discrete than when stained by the crystal violet nuclear stain.

The thionin-SO₂ procedure also gave results similar to that of the crystal violet nuclear stain but, as in acid giemsa, no cell plates were stained. The nuclei of the large cells were much larger than those of the small cells and were in many cases more discrete. Occasionally classical mitosis-like figures were observed; however, these observations were not considered sufficient evidence for such a mitosis in bacteria. In many of the large cells four, and sometimes six small rods or spots were seen, and in the small cells often two spots could be observed in cases where a 2-celled condition could be eliminated (figures 1-10). Since not all of the structures observed in the cells were in the same plane of focus, representative drawings are used to clarify the photographs. If these spots were chromosomes, for which there is little evidence, the ploidy of the large and small cells would be established. These structures could be called chromosomes but, in consideration of the present knowledge of bacterial cytology and the lack of understanding of the precise specificity and mechanism of the stain reaction, there is at present little justification in such nomenclature.

In no case was any clear evidence of "classical mitosis or meiosis" observed, and the additional stain work supported the earlier concept (Webb and Clark, 1954) that the nuclear membrane normally remains intact during nuclear division in bacteria. A similar concept has also been postulated by DeLamater (1954). This intact nuclear membrane normally obscures the internal nuclear structure and thus makes it impossible to determine the exact mechanism of division by using the presently available technics. From a genetical viewpoint the cell must undergo mitosis since there is normally an even distribution of genetic determinants to the daughter cells. However, this does not infer that a "classical cytological mitosis" is responsible for the genetic division as assumed by DeLamater (1954).

Effect of chemical and physical agents on formation. In the course of experiments to determine the conditions under which the large cells formed,

TABLE 3

The effect of ultraviolet radiations on the occurrence of large cells of Micrococcus aureus

Age of Culture at Time of Irradiation	Exposure Time	Percentage Large Cells
<i>hr</i>	<i>sec</i>	
0	0	1
0	2.5	3
0	10	15
0	15	33
0	20	45
0	30	30
0.5	20	35
1.0	20	25
4.0	20	1

it was noted that several chemicals and radiation stimulated the formation of these cells. Most of these stimulating agents have been classified as radiomimetic in that they may induce haploidization in diploid cells of *E. coli* (Lederberg *et al.*, 1951). In this case the opposite effect was found, since the agents apparently induced diploidization in haploid cells.

The effect of x-rays and ultraviolet light on the formation of large cells was investigated by irradiating surface inoculated nutrient agar dishes for varying lengths of time at different cultural ages. The dishes were then incubated at 37 C for 8 hours and the organisms checked by examining nuclear stained smears from each petri dish. As shown in table 3, a 20-second exposure to ultraviolet light at the time of inoculation produced the maximum yield of typical appearing large cells under the experimental conditions used. Irradiation of cultures $\frac{1}{2}$ to 1 hour old was less effective, but it also resulted in increased large cell formation. It was concluded that resting cells or early lag phase cells could be utilized effectively in stimulation experiments, but log phase cells were ineffective.

X-radiation at 75 kv and 15 ma at a target distance of 8 inches was ineffective in increasing the incidence of large cells. This was determined for times of radiation varying up to 4 minutes.

The effect of formaldehyde was tested by the gradient plate technic (Szybalski, 1952) and by broth cultures containing varying concentrations of formaldehyde. The surfaces of gradient plates containing 0.01 per cent formaldehyde were spread with a moderate suspension of resting cells. After 48 hr several colonies appeared in the

TABLE 4
*Effect of physical and chemical agents on
 the occurrence of large cells in
 Micrococcus aureus*

Agent	Production of Typical Large Cells	Maximum Percentage of Typical Large Cells Observed	Production of Atypical Large Cells
Ultra violet light.....	+	60	—
X-ray.....	—	0	—
Formaldehyde.....	+	100	—
Hydrogen peroxide...	—	0	—
Terramycin.....	+	20	—
Aureomycin.....	+	40	+
Bacitracin.....	+	20	—
Penicillin.....	±	5	+
Chloromycetin.....	—	10	+
Dihydrostreptomycin.....	—	0	—
Polymyxin B.....	—	0	—

edge of the zone of inhibition. These colonies were found to be composed entirely of large cells which were cytologically indistinguishable from the normal large cells. These large cells tended to revert to normal after transfer to nutrient agar.

Hydrogen peroxide was tested in the same manner as formaldehyde, but no large cells were detected under the experimental conditions used.

The effects of antibiotics were determined through the use of Difco sensitivity disks. Colonies growing inside the zone of inhibition were checked for cytological and morphological appearance through the use of the crystal violet nuclear stain and the phase contrast microscope. The action of the antibiotics listed was also observed on slide cultures with the phase contrast microscope.

Most of the cells enlarged greatly from exposure to the antibiotic; however, most cells failed to divide in the presence of the antibiotic. A few of the enlarged cells proceeded to divide, forming micro colonies of these cells. The colonies picked from zones of inhibition also represented cells dividing in the presence of the antibiotic.

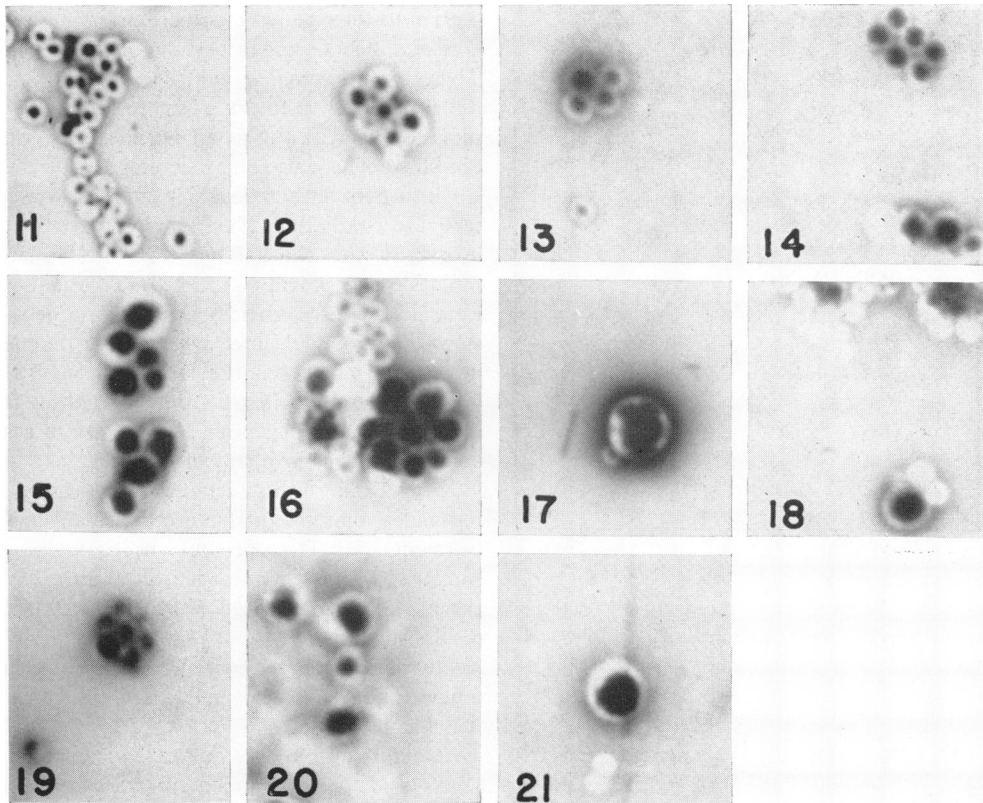
Results of these experiments are summarized in table 4. The atypical large cells produced by penicillin and chloromycetin were 2 to 4 μ in diameter with nuclei varying from a granular, lobed condition to an evenly staining spherical appearance. The significance of these forms is unknown.

The appearances of various induced large forms when stained with the crystal violet nuclear stain are shown in figures 11 to 21.

The exact relation of typical large cells produced by the chemical and physical agents with those occurring naturally has not yet been determined, although there are suggestions that in both cases a change in ploidy may be involved. It is also uncertain whether selection or induction is involved in the action of these agents since, even though the treatment apparently induces an increased formation of large cells, the environment resulting is strongly selective in favor of these cells.

Radiobiological response. Many factors have been found to influence the rate of killing of a bacterial culture when exposed to lethal radiations (unpublished experiments). These include clumping of the cells, multinuclearity, and "multicellularity." In order to eliminate these variables and thus validate interpretations of radiobiological response based on ploidy, several precautions were observed. Clumps were broken up as indicated in "Materials and Methods" and no culture was used in an irradiation experiment unless it revealed at least 90 per cent single cells. "Multicellularity" has been shown to occur in *M. aureus* and multinuclearity also can exist (Webb and Clark, 1954). The extent of "multicellularity" was checked on all suspensions by the cell wall stain and was found to be less than 1 per cent after 24 hours' incubation, and normally from 5 to 20 per cent in young cultures; however, under certain conditions the percentage was found to exceed 50. The presence of even 20 per cent such cells was found to exert no extreme effect on the final shape of the killing curve. The extent of multinuclearity was checked with the crystal violet nuclear stain and found to be negligible.

Survival curves from ultraviolet irradiation for *M. aureus* were found to be basically exponential under the usual laboratory conditions (figure 22). Age of culture was found to considerably alter the resistance to ultraviolet light, and young cells were shown to be more resistant than older cells which differs from findings previously reported (Gates, 1929). *M. aureus* has been reported as showing a sigmoidal killing rate by several investigators (Lea, 1947). Failure to eliminate clumping may be the basis of these results; a large degree of "multicellularity" would also give



Figures 11-21

Figure 11. Normal *Micrococcus aureus* cells stained with the crystal violet nuclear stain (this stain used in all photographs on this plate).

Figure 12. Normally occurring large cells of *M. aureus*.

Figure 13. Formaldehyde-induced large cells.

Figure 14. Aureomycin-induced large cells.

Figure 15. Atypical large cells caused by aureomycin.

Figures 16 and 17. Atypical large cells induced by chloromycetin.

Figure 18. Atypical large cells induced by dihydrostreptomycin.

Figures 19 and 20. Atypical cells induced by penicillin.

Figure 21. Atypical large cell induced by terramycin.

All photographs magnified 3250 \times .

rise to spurious results. It was noted that the survival curves of the 7- and 18-hour normal cells deviated slightly from the true exponential type of curve, which was probably due to the level of "multicellularity" and to the presence of dividing cells.

Large cells were produced for radiation studies under conditions which resulted in the greatest percentage of large cells together with a low level of "multicellularity." To stimulate the production of large cells, cultures were incubated at 25 C for the first 8 hours and then removed to room tem-

perature until harvested at 11 to 15 hours. Usually cultures composed of principally large cells would revert to small cells at 12 to 18 hours, giving rise to a highly "multicellular" population; thus it was necessary to microscopically examine each culture before irradiation. Under the conditions of the experiments "multicellularity" of the large cells was not found to exceed the "multicellularity" in the normal small cells of the same age, so the radiobiological response curves are directly comparable.

The survival curve for large cells is strongly

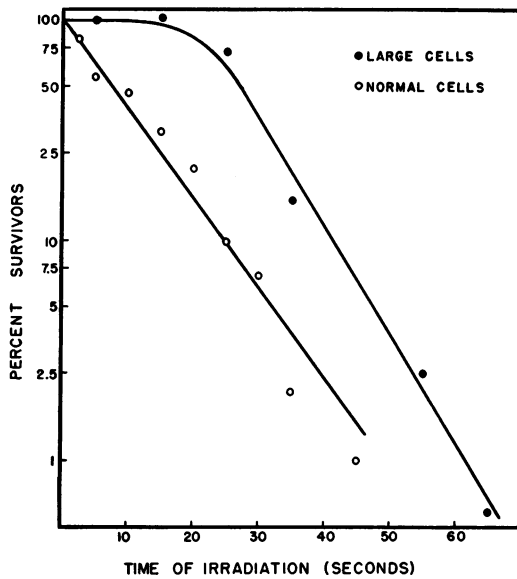


Figure 22. Ultraviolet irradiation survival response of normal and large cells of *Micrococcus aureus*.

sigmoidal (figure 22), which is typical of the "multiple hit" type of curve. The ultraviolet survival curve is approximately a 2-hit curve as compared to a hit multiplicity of 8 or more with x-ray. This together with the converging slopes of the normal killing curve and the exponential

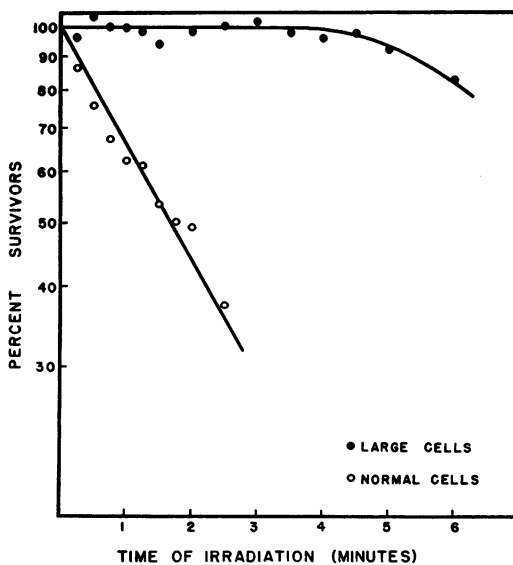


Figure 23. X-ray irradiation survival response of normal and large cells of *Micrococcus aureus*.

section of the sigmoid curve are probably indicative of a secondary effect of the ultraviolet light. These results provide evidence in addition to direct observation that the large cells are at least diploid—whereas the small normal cells, which are killed exponentially, are haploid.

Cultures of both large and small cells were subjected to x-radiation. The results were completely in accord with the findings from ultraviolet irradiation experiments (figure 23). The small cells were killed exponentially whereas large cells showed a sigmoidal survival curve. The difference in resistance between the large and small cells was considerably greater from x-radiation than ultraviolet radiation, probably because of a secondary effect of the ultraviolet light.

X-ray and ultraviolet survival curves of formaldehyde-resistant large cells were found to be sigmoidal. The survival curves were very similar to those obtained from normally occurring *M. aureus* large cells (figure 24). The results suggest that formaldehyde stimulates the production of large cells which are at least diploid and which show increased resistance to the chemical agent.

Attempts to produce an ultraviolet-resistant strain of *M. aureus* were only partially successful since all strains thus far obtained have rapidly

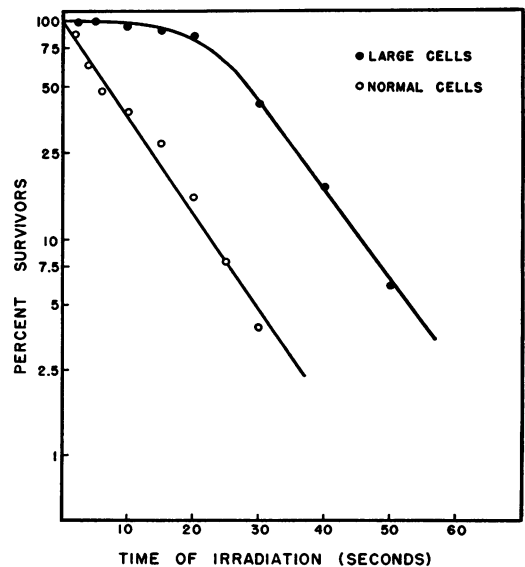


Figure 24. Ultraviolet irradiation survival response of normal and formaldehyde-resistant large cells of *Micrococcus aureus*.

reverted back to the sensitive condition. The ultraviolet survival curve of a 4-day-old resistant culture was decidedly sigmoidal and was quite similar to the survival curve of large cells. Similar results were reported in *M. albus*, in which the ultraviolet survival curves of the resistant forms were sigmoidal and the survival curves of the nonresistant forms were exponential (Rentschler and Nagy, 1942). Witkin (1947) reported that ultraviolet-resistant strains of *E. coli* strain B showed a sigmoidal survival curve in contrast to the exponential type curve for the nonresistant forms. These findings are suggestive of an association of radiation resistance and diploidy or polyploidy in many of the bacteria. However, substantiation of an association between a persistent diploid condition and radiation resistance in bacteria must await clarification of some of the conflicting reports relative to this subject.

DISCUSSION

The occurrence of life cycles in bacteria has been postulated for many years. The more significant of these references are covered by Dubos (1945) and Bisset (1950). The existence of such cycles has in many cases been based on questionable observations, and it is apparent that cyclic schemes could be postulated on the basis of morphological changes induced by varied cultural conditions or by population pressures in the developing culture. If the concept of a life cycle in microorganisms is restricted to mean those instances in which a change in ploidy is involved (with or without a proven sexual process), then there is doubt if many of the earlier observations actually involved such a cyclic event. Although much cytological evidence has been presented (Bisset, 1950) for nuclear fusion and reduction division in bacteria, there has been little supporting evidence of a physiological or genetical nature. Direct cytological observations in bacteria will be of limited value without additional supporting evidence until the mechanism of stain reactions and specificity, the significance of artifacts, and the role of fixation procedures are better understood.

The determination of ploidy in the bacterial cell cannot at present be based on cytological observations alone. In the case reported here it might be concluded that the nuclear structures sometimes observed were chromosomes and thus

a direct chromosome count would be valid. Further work may show these to be bacterial chromosomes, but proof is as yet lacking. These structures are somewhat similar to the nuclear configuration referred to as a "compound nucleus" by Knaysi (1955). Ploidy can also be determined by genetic analysis but, except in the case of *E. coli*, this is at present impossible in bacteria. The determination of the induced rate of recessive mutations would also be an indication of ploidy. In most cases of suspected or proven diploidy in bacteria, it has been found that the diploid state is unstable and the mutagenic treatment causes increased segregation (Lederberg *et al.*, 1951; unpublished data). This in addition to the complicating factors of "multicellularity," clumping, and multinuclearity can obscure the expression of a recessive allele.

Radiobiological lethal response can also be of aid in ploidy determinations. If the lethal action of ultraviolet light or x-ray is due primarily to lethal mutation or some similar single mechanism, then a haploid cell would give a survivor incidence that is exponential in relation to dose (Zirkle, 1952; Tobias, 1952). This assumes that only one "hit" or "event" at any of several sensitive sites would result in death of the cell. Since lethal mutations have been found to be recessive in many forms of life, it can be considered that such would also be the case in bacteria and the survivor response would be indicated by a sigmoidal or multi-hit curve in a diploid cell. The number of hits required in a diploid cell could be as small as 2 if the lethal action involves only chromosomal hits or if excessive segregation occurred, or it could be a large number if only recessive lethal alleles were involved. The polyploid series of yeasts has correlated nicely with killing curve shape (Latarjet, 1952), and it appears reasonable that bacterial cells should behave similarly. Unfortunately several factors can effect the shape of a killing curve and, although these are being investigated at present, their full significance is as yet unknown. A "multicellular" condition of a haploid cell would yield a sigmoidal response to lethal radiations since the technics used are based on the single-celled entity (Clark and Webb, 1955b). Similarly a multinuclear state of haploid nuclei would also show a hit multiplicity of at least 2 (Atwood and Norman, 1949). Therefore precautions were taken in these experiments to make sure that cultures con-

sisting predominantly of single-celled, single-nucleated units were used. This aided in validating interpretation of survival curves in terms of ploidy.

The interpretation of radiation-induced events which lead to inhibition of cell division in terms of genetic entities within the cell does not depend on the formulation of any theory as to the possible direct or indirect action of the radiation. The kinetics of the determined reactions is dependent on the number of events which occur and for present purposes is independent of the precise nature of this event. It could be the hitting of a specific target area within the cell; it could also be the end result of a chain of reactions occurring within the cell.

Although it has not been established definitely that the large cell of *M. aureus* is a result of an increase in ploidy, all confirming tests performed so far indicate this to be the case. Thus the interpretation of cytological evidence is strengthened by confirming physiological and genetical experiments.

The predominant appearance of these large cells in the lag phase of growth suggests that these might be the normal enlarged cell usually associated with such growth phase. However, care must be taken in distinguishing between the type of large cell reported here which involves a definite nuclear change and the type of enlarged cell which results from protoplasmic synthesis exceeding the rate of cell division. The large cells involving apparent ploidy change in many cases persist long after the lag phase of growth.

In dealing with large populations of cells such as found in a bacterial culture, a homogeneity of cells cannot be assumed. Cytological investigation on the large cells of *M. aureus* reveals several types of structures which could correspond to haploid, diploid, and tetraploid cells, all within the same culture. Thus the terms diploid or polyploid are not used in the strict sense of their specific meaning when used in referring to the ploidy conditions of a culture. To overcome the problem of incorrectly using well-established terminology, we propose that the term, mixoploid, be used in referring to such cultures. A condition of mixoploidy would refer to a population of cells in which a mixture of ploidy occurred with the majority of cells being diploid or greater in ploidy.

SUMMARY

The large cells of the apparent life cycle of *Micrococcus aureus* were investigated, and the conditions were found under which a large percentage of such cells formed. Cytological studies with various stains and with phase microscopy did not reveal the precise origin of the large cells, but information was found which suggested an intracellular nuclear fusion to yield a diploid or polyploid nucleus which would then undergo meiosis and result again in normal haploid cells. The diploid nature of the large cells was substantiated by radiobiological studies. In these, the dose-survivor relationship was exponential in the normal cells with both x-ray or ultraviolet radiations. This single-hit type of response can be interpreted as a criterion of haploidy. The dose-survivor curve of the large cells when irradiated by x-ray or ultraviolet light was sigmoidal, showing a hit-multiplicity of two or more—thus indicating the diploid or polyploid nature of these cells. Factors of clumping, multinuclearity, and multicellularity were investigated in order to validate ploidy interpretations of the radiobiological responses.

It was found that ultraviolet light and certain chemicals would cause an increased incidence in the large cells. In many cases the large cells thus produced were indistinguishable from those occurring naturally, but some of the chemicals tested caused atypical-appearing large cells to form.

The appearance of the large cells was found to be a fairly generalized phenomenon, since they were found in most of the coccus cultures which were tested. Thus the existence of a simple life cycle involving a ploidy change may be common in the bacteria.

It was suggested that the term, mixoploid, be used in referring to populations such as bacterial cultures in which a mixture of ploidy is found with the majority of the cells diploid or greater.

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