BACTERIAL VARIATION: AN INQUIRY INTO THE UNDERLYING PRINCIPLES GOVERNING THE CELL MORPHOLOGY OF BACILLUS MEGATHERIUM

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In a recent publication (1933) we discussed briefly the pronounced influence of environment upon cellular morphology in certain stock strains of *Bacillus megatherium*. The present investigation marks a continuation of our earlier study and attempts to throw light on the significance of the variant cell forms so frequently seen in *Bacillus megatherium* cultures, by inquiring into some of the more important environmental factors which govern their existence.

It is unfortunate that nearly all of the reports published in support of the theory of complex bacterial life cycles have rested largely upon evidence obtained from the study of non-living organisms in stained preparations. For many of us such studies cannot supply convincing evidence concerning the developmental or cyclic relations existing between cell variants. Furthermore, our observations on the very irregular distribution of variant cell types throughout a given colony cast additional suspicion upon any hypothesis that depends for its support on the examination of smears made at random from old cultures. In order to avoid fallacies that are inherent in the stained-smear method, we have worked during this entire investigation with living cells and have followed their formation and continued development, under the microscope. *B. megatherium*, because of its large size, lends itself admirably to this type of study.

MEDIUM AND METHODS

Plain nutrient agar containing 0.3 per cent Bacto-meat extract, 1 per cent Bacto-peptone and 1.8 per cent Bacto-agar, and

having a final reaction of pH 6.8 to 7.0, was employed as the basal medium throughout this investigation. It was sterilized in test tubes containing about 12 cc. each, and was clarified by high speed centrifugation immediately before use.

Our first attempts at microculture study were made on the tips of agar cones built up in deep-well depression slides, according to the method of Adolph and Bayne-Jones (1932). The hanging block device of Hill (1902), with certain modifications, was soon adopted, however, because it offered definite advantages when cultures were to be incubated and observed over long periods of time.

Preparation of hanging block cultures

Sterile nutrient agar (5 to 9 cc., according to the depth desired) was pipetted, after centrifugation, into Petri dishes and allowed to solidify. Blocks 1 cm. square were cut out of the solidified agar and after inoculation were placed on 22 x 50 mm. sterile coverslips, with their inoculated faces against the glass. They were fixed firmly in place on the coverglasses by running melted paraffin along their edges. The coverglass preparations were then inverted over rectangular moist chambers (6 x 22 x 50 mm.), and sealed to the supporting walls of the latter with paraffin. Chambers deeper than 6 mm. were not employed, because they would have necessitated the use of an unusually intense source of illumination. Undue exposure of cultures to the light was avoided. During use, the open end of the rectangular chambers was temporarily sealed with plasticine.

It was unnecessary and, in fact, inadvisable to introduce water into hanging block chambers except when they were opened to permit some aeration; at such times sufficient water was added to compensate for the moisture lost through evaporation. With proper care and manipulation microcultures could be kept under observation for several weeks.

Throughout these experiments the temperature of incubation was 30°C. This temperature was maintained during continuous microscopic observation by enclosing all but the barrels of the microscopes in specially built "incubator boxes." At other times

moist chamber cultures, or microscopes with microcultures in place, were held in an ordinary incubator, and removed to incubator boxes when temporary microscopic observations were made.

Bacterial cell outlines were traced with the aid of a Zeiss camera lucida, at a magnification of approximately 2100 diameters. A 20× compensating eye piece and a $\frac{1}{12}$-inch oil immersion objective were employed for drawing. In order to avoid overlooking important microscopic details, all bacterial cells were examined, after tracing, with 8×, 10× or 12× oculars.

Departures from the procedure described above were frequently made necessary by the nature of special problems. For example, early in our work we observed that cellular variation in *B. megatherium* was definitely influenced by associated growth, but that the massing of cells necessary to bring out variant types interfered with the optical conditions required for detailed microscopic examination. To obviate this difficulty, the following technique was employed.

A light inoculum was placed on the upper or coverglass surface of an agar block, its density being determined by the number of microcolonies desired. After the block had been sealed to the coverslip, and immediately before it was suspended over a micro-chamber, its free surface (lower surface in the chamber) was inoculated with the same organism by streaking over its entire area or any selected portion thereof. The underlying colonies thus provided served as associated growth during the experiment and exerted a very marked influence on the overlying test culture. In order to prevent the underlying culture from becoming so dense as to cut down markedly the light from the sub-stage lamp, a second sterile agar block was placed over the first, thus sandwiching in the underlying inoculum and causing it to develop in the form of a thin film, rather than as dense, thickly layered surface growth. This growth did not interfere with optical conditions, because it lay in a widely different optical plane.

At first, suspensions containing varying numbers of bacterial cells were placed upon the two surfaces of the primary hanging block. Colony localization and the relative positions of overly-

ing and underlying colonies were, with the use of this technique, matters of chance. We later found it possible, by employing moderately dry agar, to follow definite inoculation patterns, and thus to control to a very large extent the conditions required for satisfactory study. For example, the overlying growth was sometimes made to consist of a single colony at the center of the block, and the underlying growth distributed over one-half of the block's inferior surface, leaving the other half free; or, at other times, the underlying growth was made to develop as a relatively narrow ring beyond the border of the overlying colony. Inoculation designs could in this way be suited to the purposes of different types of experimentation (see figure 27, plate 1).

THE INFLUENCE OF SURROUNDING GROWTH ON CELLULAR VARIATION

Early in our investigation we observed that rather heavily inoculated microcultures rapidly developed variant cell forms (in from one to three days), and that such forms appeared first in the sections of colony peripheries which lay most nearly adjacent to other colonies. Variation in such cultures was quite extreme and gave rise to cells ranging in shape from long, branched and unbranched filaments, through tapering and clubbed rods to short cubical and globular cells occurring in chains. The predominating cell type in any section of a colony appeared to be directly related to the density of population around that point. On very lightly inoculated agar blocks, on the other hand, cellular variants appeared late in the history of the culture, were never striking in form, and developed in horizontal strata deep within the cell mass. The layer directly under the coverglass was composed of moderately long, uniform rods which quickly became granular and never showed any further development, but gradually autolyzed and disappeared. Their rapid death was probably due to the fact that they were cut off, by the cells lying between them and the agar block, from their supply of nutrients and oxygen. The layer nearest the agar, because it was able to continue its growth under unfavorable conditions for the longest time, showed the most complete development of coccoid

cells. Under such conditions peripheral cells developed normally for as long as eleven days.

In order to explain the observed outstanding differences in time of appearance and localization of variant cell forms on crowded, as compared to thinly seeded blocks, it is necessary to postulate some "variation-exciting" influence or group of influences associated with density of population. It seems reasonable to suppose that certain of the environmental changes which follow metabolic activity as, for example, altered H-ion concentration, oxidation-reduction potential (or free oxygen supply), decreased nutrients and increased metabolic products, may play a major rôle in stimulating cellular variation under the conditions of these experiments. Such variation-determining factors are, naturally, most concentrated at centers of population (colony centers) and gradually decrease in potency as they diffuse out through the hitherto unattacked medium. They become more pronounced as the cultures grow older and more luxuriant, and they accumulate more rapidly on crowded than on thinly populated blocks. Furthermore, they are particularly accentuated where the zones of influence of two or more colonies overlap. In high concentrations they appear to be definitely unfavorable to cell development. At somewhat lower concentrations, however, while still slowing down development, these factors seem actively to stimulate cellular variation. Cruickshank (1934) has offered a very interesting demonstration of the influence of such factors on bacterial luminescence.

On very thinly populated blocks these variation-stimulating influences accumulate slowly, and never become sufficiently concentrated at colony peripheries to influence cell morphology. At the centers of even well-isolated colonies, however, they do gradually increase in concentration and eventually cause the development of coccoid and globular forms. On densely populated blocks, on the other hand, they accumulate much more rapidly and growth at colony centers may be stopped so quickly as to prevent cell variation entirely. The peripheral cells on crowded blocks feel these unfavorable influences more slowly than do those within the colonies; but even there these environ-

mental changes soon become so marked as to produce striking variations in cell morphology, followed by gradual cessation of growth. Thus, cellular variation may be observed to occur in those parts of a culture where there exists a definite balance between factors that are unfavorable and those which are favorable to growth. Variation appears here to be the result of an attempt on the part of developing organisms to adapt themselves to unfavorable environmental conditions.

Because the cell crowding necessary to induce variant forms interfered to some extent with the optical conditions required for clear microscopic observation, the modified hanging block technique described above (page 212) was devised. The results obtained with this method agreed entirely with those just reported. If the inoculum on the inferior agar surface was large enough to result in solid and continuous growth over its entire area, no marked variation in the cells of the overlying culture took place. On four out of six culture blocks having heavy underlying growth, only delicate wisp-like colonies developed which were composed of "normal" rods. On the other two the lower growth was less dense and a few coccoid and wedge-shaped cells were seen in the overlying culture. No further changes took place, aside from granulation of the cells, because unfavorable factors accumulated so rapidly in the medium as to cause complete cessation of growth in the overlying culture before material changes in morphology had time to develop. The analogy existing between such cells and those located at the centers of colonies on thickly populated blocks (without underlying growth) is quite apparent.

With greatly decreased concentration and irregular distribution of the underlying growth, overlying cultures were able to continue their development for longer periods of time under *relatively* unfavorable environmental conditions. Striking variations in colony size and cellular morphology appeared in such microcultures. For example, during the first twelve to fourteen hours of incubation on a relatively crowded block the cells in the overlying culture developed normally, although many of them were somewhat shorter than those in well-isolated colonies of the same age. Later, numerous short, pointed or wedge-shaped

cells appeared at colony peripheries. By the sixtieth hour some microcolonies contained rather long, thin, sharply pointed cells in their borders, while other peripheries showed coccoid cells and shorter wedge-forms. Other colony edges revealed only normal rods.

After ninety hours many variant cell types developed in different sections of the block. Clubbed and pointed forms were quite common and ranged in size from extremely long thick cells down to cells so short that their length scarcely exceeded their thickness.

In one of our preparations, after one hundred and ten hours' incubation the probable function of the "runner-type" cell (see page 227) was clearly revealed. In several instances such cells carried growth out beyond their colony of origin to less densely populated areas, there to establish new colony units. The new microcolonies contained apparently normal cells, and the "runner" cells underwent autolysis after accomplishing their purpose. Their faint outlines could be seen for some time, however, stretching between mother and daughter colonies.

Occasionally a wide zone of very large long-handled clubs and long thick rods was seen developing beyond a ring of small wedge-shaped cells. Some of the clubbed cells had equally large swellings at both ends. Fringes of long, branched filaments later developed beyond the rings of clubbed cells. Long "rope-twists" or filaments wound around each other were also prominent at times. Autolysis, which was always observed in certain sections of aging cultures, became more and more pronounced, and was quite marked at the end of one hundred thirty-six hours.

Practically all of the cells having unusual morphology were hyaline and had the general appearance of being viable. Although they were often seen lying in masses of autolyzed cellular debris, they apparently resisted autolysis. Furthermore, they were too numerous to be the result of mere chance.

The relation between the morphology of the overlying cells and the concentration of the underlying growth was clearly apparent. The length and tapering tendency of wedge-shaped cells and the length of the "handles" of clubbed forms varied

inversely with the relative density of the lower (associated) growth.

In an attempt to control the distribution of the underlying growth and its relation to the overlying culture, six hanging blocks were set up. The overlying growth on each of them was made to consist of a single colony at the center of the block. One inoculated block, destined to serve as a control, received no underlying inoculum. Graded concentrations of a suspension of *B. megatherium* were streaked over one-half of the lower surface of each of the five remaining blocks, leaving the other half uninoculated. Figure 27, plate 1, shows the relative locations of overlying and underlying growths. During the entire period of observation (seventeen days), that portion of the overlying colonies which extended out over underlying growth (V) revealed essentially the same cellular changes as were observed in earlier experiments in colonies developing over heavy underlying growth; i.e., at the end of *fourteen hours* the peripheral cells in this section of the colony were already beginning to become globular. Later, clusters and fringes consisting largely of these globular forms developed. Such cells as were unable to effect an appreciable change from the original rod form became very granular and underwent autolysis. The peripheral cells on the opposite sides of the colonies, however, remained normal in appearance during the first five days. On the *sixth day* several long-handled clubs were seen developing at "X," a cleft formed where two parts of one of the colonies came together. By the *seventh day* many clubbed forms had appeared. They were rather generally distributed through the border cells, but seemed to be particularly numerous at "X," where they were first seen. On the *eighth day* many clubbed forms were still visible, but a fringe of long filaments had developed beyond them. Many pairs of these filaments had begun to twist themselves in rope-cord fashion. They continued to develop slowly from the *ninth* to the *seventeenth day*. Each rope or braid was made up of two twining filaments.

It was interesting to note that throughout these six experiments the different types of cellular variants appeared in rather definite sequence: first normal rods, then clubbed and pointed forms,

followed by thin long filaments, branched forms, and finally twisting filaments. This chronological appearance of variants, however, does not necessarily indicate the existence of a cyclic relationship between the various cell forms observed. One variant form was never seen to develop into another variant type; for example, although long branching filaments followed clubbed forms in their appearance in aging colonies, the former did not develop from the latter, but from "normal" rods which existed among the clubbed cells. "Normal" rods under varying environmental conditions developed into: (1) coccoid or globular cells; (2) wedge-shaped cells of different lengths; (3) clubbed cells of various lengths and (4) filaments of various lengths, twisted or straight, and with or without branching. Some of these variant cells were able to develop back into "normal" rods when they were transferred to a fresh medium. Their apparent cyclic occurrence was, we believe, in reality due to the progressive environmental changes which occurred in the medium on which they were developing, rather than to any inherent cyclic tendency in the species. Each type of cell came out in response to definite, delicately balanced environmental conditions.

THE INFLUENCE OF OXYGEN STARVATION ON THE CELLULAR MORPHOLOGY OF B. MEGATHERIUM

As a result of early indications that free oxygen may play a significant rôle in the development of variant cells, an attempt was made to study the effect of mechanical oxygen exclusion on cellular morphology. Hanging block cultures were prepared in the usual manner except that the lower surface of each block was entirely covered by a coverglass of sufficient size to project slightly beyond the edges of the agar. The space around the agar between the overlapping edges of this glass and the regular coverglass was filled with paraffin, thus completely sealing in the block. Minute amounts of atmospheric oxygen penetrated to the culture through the paraffin seal, but they were far too small to allow continued normal growth. Under such conditions colonies at first developed normally. Soon, however, as the oxygen supply approached depletion, due to its rapid utilization, the periph-

eral cells became shorter. After a further period, the length of which depended upon the number of colonies developing and the thickness of the agar block, all of the peripheral cells were coccoid or globular. In appearance the microcolonies resembled those which had developed over very heavy underlying growth. (Fig. 1, Plate 2).

Certain globular cells appeared to be less susceptible to the unfavorable influence of reduced oxygen supply than were others. They continued to develop slowly for some time after general growth had ceased, and formed clumps of globular bodies, some of which remained viable and unautolyzed for many days after all of the rod-shaped cells surrounding them had died and autolyzed. Such globular cells correspond, we believe, to the "round regenerative bodies" reported by Löhnis and Smith (1916 and 1923). The autolyzing cellular débris in which they appear closely resembles Löhnis' "symplasm." However, we can safely conclude that our globular cells developed, not from the so-called "symplasm," but from normal rods. The exact manner of their formation has been studied, and their reversion to normal rods has been repeatedly observed (see page 219). Although they were made to appear by the simple mechanical exclusion of oxygen, they resembled fully the globular forms reported above as arising under the influence of "associated" growth. (Figs. 3, 4 and 5, plate 2).

When free oxygen was re-admitted to sealed cultures by removing the lower coverglass, the relatively resistant globular cells quickly resumed development and their progeny soon reverted to long, hyaline rods. It appears that the globular cells in *B. megatherium* cultures may serve to carry the species through certain unfavorable conditions, thus supplementing the function performed by spores. This phenomenon may have special significance in so far as *B. megatherium* is concerned, since it is well known that its cells cannot produce spores under materially reduced oxygen tension (Bayne-Jones and Petrilli, 1933).

Resistant globular cells were invariably developed under conditions of reduced oxygen tension, providing the oxygen supply was not too suddenly or completely depleted. The number of

globular forms appearing in a culture was, in general, inversely proportional to the speed with which oxygen was exhausted. When the oxygen supply was cut off very rapidly and strict anaerobic conditions were maintained, growth ceased before cellular variation could take place, and all of the normal rods in the culture soon died.

Whether such globular cells, when formed, can live for an appreciable period of time in a definitely anaerobic environment has not yet been determined. It seems quite probable, however, that their viability under greatly reduced oxygen tension is at least much greater than that of "normal" rods.

RE-STIMULATION OF GROWTH WITH POTASSIUM PERMANGANATE

Since we had observed repeatedly that re-stimulation occurred at once when atmospheric oxygen was re-admitted to cultures the growth of which had been interrupted by its mechanical exclusion, and since mechanical exclusion of oxygen always resulted in the development of colonial and cellular types similar to those which were formed over heavy underlying growth, it seemed that re-stimulation should occur in overlying cultures if more oxygen could be supplied to them.

Oxygen could not be mechanically admitted to such cultures because it had been excluded biologically, through the oxygen demand of underlying cells. Hence, to impart a distinct oxidative character to hanging block cultures, various oxidizing agents were employed, namely potassium permanganate, hydrogen peroxide, potassium chlorate, sodium nitrate and methylene blue. These were applied to the lower surfaces of hanging block cultures in which growth had ceased due to crowding. Striking results have been obtained thus far only with potassium permanganate.

When a crystal of potassium permanganate was placed in a small drop of water on one corner of the lower surface of a hanging block culture in which growth had ceased, and was allowed to remain on the agar until the color of the permanganate had diffused out over one-fourth of the block, the growth of normal rods was stimulated where the medium was faintly colored and

for a short distance beyond that area. Colonies farther away from the $KMnO_4$ region did not change. Ninety-eight hours after the application of permanganate many long-handled clubs and very large, long tapering rods could be seen in areas just beyond the zones colored by the permanaganate. The parts of colonies that were just within the colored area contained "normal" rods.

A full explanation for the growth re-stimulating influence of $KMnO_4$ is not, as yet, at hand. Several factors may be involved, but it appears probable that the positive effects obtained depended on the ability of $KMnO_4$ to furnish available oxygen to the culture, since its action was like that of simple atmospheric oxygen when air was re-admitted to cultures from which it had been mechanically excluded. From the fact that normal rods developed in areas that were faintly colored by the permanganate, and that clubbed and tapering forms appeared immediately outside of this zone, in an area between that in which normal rod forms had been re-stimulated and that in which no renewed growth occurred, we were led to assume that a certain approach to oxygen starvation stimulated the development of tapering and clubbed cells. Of course, it is also possible that the permanganate in high concentration completely destroyed some variation-stimulating metabolic product or products which were only partly destroyed by lower concentrations. This phase of the investigation is being carried forward, and we hope that it may be possible, by regulating the amount of oxygen available, to control the type and degree of cellular variation.

LOCALIZATION OF VARIANT CELLS, AND ITS RELATION TO THE INTERPRETATION OF DATA OBTAINED FROM STAINED PREPARATIONS

During our investigation we found that morphological variants did not appear uniformly distributed in colonies, but occurred in definitely circumscribed areas. Three types of localization were observed: (1) horizontal stratification; (2) peripheral zone formation, and (3) clump or cluster formation.

The first type (horizontal stratification) was observed in well-

isolated colonies where, as we have pointed out, striking cell variants never appeared. In such colonies the centrally located cells gradually decreased in length, with repeated multiplication, some of them actually assuming globular form. Cells of varying length were definitely segregated into horizontal layers.

That stratification is not the result of microcultivation but occurs also in cultures aging under normal conditions, is indicated by the work of Legroux and Magrou (1920). Using histological procedures, they cut and stained cross sections of cholera colonies and demonstrated definite morphological and tinctorial strata. Kahn and Nonidez (1933), also using histological methods, found three sharply differentiated layers in old colonies of *Mycobacterium tuberculosis*. These layers varied in acid-fastness, the surface zone being made up almost entirely of non-acid-fast rods and granules, and the deepest layer of acid-fast cells. The authors believed that the surface zone was the youngest layer of the colony. It appears, however, from our experience with microcultures of *B. megatherium* that the opposite may be true.

The second type of localization (peripheral zone formation) occurred very frequently. The more extreme cell variants (branched, clubbed, pointed, etc.) nearly always developed, under the conditions of our experiments, in narrow fringes around the edges of colonies. This made microscopic observation and study of the development of such cells particularly easy.

Globular cells often showed the third type of localization. Under certain conditions (relatively slow oxygen removal) microcolonies were studded with many clusters of large coccoid cells. With more rapid oxygen removal fewer clumps of globular cells developed. Sometimes only one or two clusters appeared in a colony; the remainder of the cell mass slowly autolyzed. (Figs. 3, 4 and 5, plate 2).

Because morphological types are not uniformly distributed throughout aging colonies but, as we have shown, often occur at certain points in high concentration, ordinary microscopic slide mounts from such colones may fail to present reliable cross-sections of the colony population as a whole. Indeed, it is quite possible that, in making smears from an aging colony by the

customary procedure, one may at one time pick up only a purely amorphous mass of cellular debris, while at another time one's needle may happen to touch a portion of the culture which contains an appreciable number of cells of some definite morphological type. It seems clear that one should not assume from such an observation that the cellular forms seen in the second preparation developed from the amorphous material observed in the first; yet, much of the work supporting the theory of complex bacterial life cycles (including amorphous or symplastic stages) has rested entirely on the study of stained smears. Our attempts to observe in microcultures the development of cellular forms from amorphous matter have thus far given negative results. Wyckoff (1933 and 1934) reported similar failures with other organisms.

THE ORIGIN AND FURTHER DEVELOPMENT OF CERTAIN VARIANT CELL FORMS

Very few attempts have been made to study the progressive morphological changes which occur in undisturbed cells as they develop in an aging colony. Most investigators who have reported work with microcultures have stated that after a few hours' incubation these cultures became so crowded as to render them useless for further study. We have found, however, that such is not necessarily the case, and that observations on isolated individual cells can and should be supplemented by investigations on the morphology of cells living in developing colonies. Cells growing in an undisturbed colony are as nearly in their "common or customary" environment as it is possible for them to be while under observation. It seems that, by starting with a single isolated cell and following its progeny for many days under the microscope, we should be able to obtain a reasonably accurate picture of the life history of the species in question. This should be particularly true if microcultures are grown under varied environmental conditions. Certainly, such procedures are open to fewer criticisms than are the other methods which are available at the present time.

During the early rapid growth period it is, of course, impossible

to follow the development of any particular cell and its progeny for an appreciable time. This is not a serious drawback, however, since frequent observations have shown us that practically no morphological variation occurs in *B. megatherium* cultures during this period. Hill (1902) stated that branching may begin in a culture of *Corynebacterium diphtheriae* within five or six hours after inoculation. Gardner (1925), working with seven species, reported that early growth in some cases was rich in "Y" forms; and Wyckoff (1934) stated that young Mycobacterium cultures may show many branching cells. It is possible that some of these observations may be accounted for by the fact that branched organisms develop more slowly than do normal rods, and that when they are carried over with the original inoculum they may remain dormant on the block, and be visible for hours lying among the normal, developing rods.

Pleomorphic cells begin to form in microcolonies of *B. megatherium* long after the logarithmic growth period has past. They often develop at colony peripheries, because further back in the colony growth has already ceased. This tendency toward peripheral localization makes microscopic study of their formation and development particularly easy. Growth at this time is so slow that repeated camera-lucida drawings can be made.

We wish again to emphasize the importance of direct microscopic study of living cells and their development in the particular environment that called them forth. Lewis (1932), proceeding on this principle, but using a less specialized technique, gained a very interesting insight into the significance of certain variant cells of *Bacillus mycoides*. Ørskov (1927) tried to study the growth cycle of the pleuropneumonia organism by observing development at the edges of young cultures on a solid medium. These attempts, we believe, represent steps in the right direction. Only by studies of a similar nature can we hope eventually to unsnarl the mass of conflicting evidence bearing on bacterial pleomorphism and its significance.

Under certain relatively unfavorable cultural conditions (oxygen starvation, for example) growth and reproduction are both slowed down. But growth in size is apparently more sensitive

to these unfavorable influences than is cell division. When the rate of cell division is more rapid than the rate of growth in cell size, cells become progressively shorter as reproduction continues. This phenomenon, carried to its extreme, results in the formation of cells which are no longer than they are broad (see figures 1, 3, 4 and 5, plate 1). Cells which have reached this state of extreme shortness tend to round up and become globular, resulting in the coccoid and yeast-like forms which have so often been reported as occurring in the reproductive cycle of many bacillary species. Our data indicate, however, that they are not formed as the result of any unusual or specialized reproductive mechanism, but that they are the result of the repeated binary fission of rods whose growth in length has been greatly retarded. Wyckoff and Smithburn (1933), Wyckoff (1934) and Lewis (1932) have also reported this phenomenon in other species of bacteria. The globular bodies that develop in this way are more resistant to the increasingly unfavorable environmental conditions that cause them to develop than are "normal" rod forms. They can be seen in old colonies lying in masses of autolyzing cellular debris (figures 3, 4 and 5, plate 3), and resemble, we believe, the "round regenerative bodies" reported by Löhnis and Smith (1916, 1923).

We have been able to observe the "regeneration" of these round yeast-like cells under the microscope (see figures 6 through 17, plate 1). When placed on fresh agar, they return to "normal" rod forms by a reversal of the process which led to their formation. Growth in cell length occurs more rapidly than does cell division. After four or five divisions the culture is again made up of rods of normal length. Soon after globular cells are placed on fresh agar they begin to swell, but often they do not do so uniformly on all sides. At first one side of the cell stretches out more rapidly than the other, giving a curved, bean-shaped appearance to the organism. It is probable that the cell wall of the older globular forms becomes somewhat thickened or hardened. After one or two divisions, however, this difference is no longer apparent.

A second type of round cell was repeatedly observed in cultures of *Bacillus megatherium*. Similar forms have been reported by

other investigators as occurring in several species of bacteria, and have been referred to as buds, gemmules, conidia or gonidia.[1] In our microcultures these cells did not form by budding, but by fission (see figures 19 through 21, plate 1). The division occurred, however, not at the center of the cell, but well towards its distal end (see figures 20 and 26, plate 1). Under certain conditions such round end balls were seen to elongate slowly and to return to the normal rod form (see figures 21 through 25, plate 1). The rods which developed from round terminal cells were never seen to divide; the significance to the species of such cells is not yet known. They differ from the large globular yeast-like forms in that the end balls develop very early in the history of a colony, while the other globular cells appear much later.

In certain cultures we have observed an interesting cell type which grows very rapidly in length. It differs from the average filament in that it tapers gradually toward its distal end (see figure 18, plate 1). We have called it the "runner type" of cell, because it runs out from the colony of origin, outdistancing the cells which surround it. Its apparent function is that of carrying growth out beyond the unfavorable surroundings of the mother colony to fresh "soil." When the tip of such a cell gets well away from the mother colony it begins to divide, and a new colony develops from the cells arising from it.

DISCUSSION

The more or less indiscriminate use of the term "life cycle" has led to much confusion in the minds of bacteriologists. To some it signified changes in cell morphology which include pleomorphism and complicated reproductive processes, some of which (we believe) may not be essential to the permanent existence of the species. To others it implies morphological changes through which an organism must pass from any given stage back to the same stage as, for example, from the egg to the egg, or from the butterfly to the butterfly.

[1] The recent observations of Rudakov (1934) are of particular interest. He noted such globular forms in a member of the *Lactobacillus* genus and reported them as filterable.

Bacteria, in common with all of the higher forms of life, pass through a cyclic developmental process from generation to generation, however simple this may be as compared with the transformations through which many protozoal parasites go, to which the term "life cycle" must and does apply. The present controversy on variation and life cycle hinges, therefore, not on the existence of such a thing as a life cycle, but on the types or phases through which bacteria pass as a part of their life cycle. For example, does the passing of *Bacillus megatherium* from rod-to-rod-to-rod, with certain variation in cell size, constitute its life cycle? Or does the cycle include the sporulation phase, as we have long assumed? Or, finally, must we include under this term the various branched, clubbed, filamentous and coccoid cells which may be seen in cultures under certain conditions?

If cells of varied, and often of extreme, morphology represent real stages in the life cycle of the species in which they appear, and are not merely variant forms which arise in response to changing environment, it should be possible to show (1) that they possess full vigor, (2) that they develop from each other in more or less definite sequence, and (3) that they are essential for the maintenance of the species in full vigor.

We need not look far for variants among the higher forms of plant and animal life which have become such in response to certain environmental influences. They represent attempts at adaptation. The misshapen and bent-over pines on the sea coast or open arid plains which struggle for existence against strong trade winds, the dwarfed and scrubby oaks that grow on mountain sides above the tree line, and the long, scraggy yellow grass found underneath log or rock piles, are all examples of this type of variation. Although they constitute distinct departures from the normal, they cannot in any sense be regarded as phases of a life cycle. They represent last attempts of individuals to maintain themselves under unfavorable environmental conditions. There is, we believe, a striking analogy between such forms and the variant cells which we have observed in cultures of *Bacillus megatherium*.

SUMMARY

1. The cell morphology of *Bacillus megatherium* is governed largely by environmental conditions. Relatively slight changes in environment are responsible for striking changes in cell form.

2. Cellular variation can be related directly to the degree of growth crowding and, consequently, to changes which take place in the medium as a result of increasing metabolic activity.

3. The factors which stimulate cellular variation are apparently unfavorable to continued normal growth. When they accumulate very rapidly in a culture development ceases before variation can take place. In other words, variation is possible only when favorable and unfavorable influences are so balanced as to permit slow growth in the face of untoward circumstances.

4. Partial oxygen starvation has been shown by us to be directly responsible for at least certain types of cell variation.

5. It appears that some variant cells are more able to resist the unfavorable conditions that call them forth than are the "normal" rods, and hence may be regarded as adaptation forms which aid in the preservation of the species.

6. We have had no evidence as yet which would indicate that definite cellular forms develop from finely granular or apparently structureless material. We wish to stress again the importance of subjecting cultural material to rigid and continued microscopic examination in life cycle studies.

REFERENCES

ADOLPH, EDWARD F., AND BAYNE-JONES, STANHOPE 1932 Jour. Cellular and Comp. Physiology, **1**, 387–407.

BAYNE-JONES, STANHOPE AND PETRILLI, ALEXANDER 1933 Jour. Bact., **25**, 261–276.

CRUICKSHANK, J. 1934 Jour. Path. and Bact., **39**, 141–148.

GARDNER, A. D. 1925 Jour. Path. and Bact., **28**, 189–194.

HILL, HIBBERT W. 1902 Jour. Med. Res., **7**, 115–127.

KAHN, MORTON C., AND NONIDEZ, JOSE F. 1933 Proc. Soc. Exp. Biol. and Med., **30**, 577–582.

KNAYSI, GEORGES 1933 Jour. Bact., **26**, 623–644.

LEGROUX, RENE, AND MAGROU, J. 1920 Annales de l'Institut Pasteur, **34**, 417–431.

LEWIS, I. M. 1932 Jour. Bact., **24**, 381–414.

LÖHNIS, F., AND SMITH, N. R. 1916 Jour. Agric. Res., **6**, 675–702.

LÖHNIS, F., AND SMITH, N. R. 1923 Jour. Agric. Res., **23**, 401–432.
ØRSKOV, J. 1927 Annales de l'Institut Pasteur, **41**, 473–482.
RETTGER, LEO F., AND GILLESPIE, HAZEL B. 1933 Jour. Bact., **26**, 289–318.
RUDAKOV, K. J. 1934 Centralbl. f. Bakt., II Abt., **90**, 67–74.
WYCKOFF, RALPH W. G. 1933 Jour. Exper. Med., **57**, 165–180.
WYCKOFF, RALPH W. G. 1934 Jour. Exper. Med., **59**, 381–392.

PLATE 1

With the exception of figure 27, the illustrations in this plate represent camera lucida drawings of living cells of *B. megatherium* developing on extract agar hanging blocks. ×1000.

FIG. 1. Chain of globoid cells from hanging block culture which had been held under partial anaerobic conditions for nineteen days.

FIG. 2. Embryonic cells of *B. megatherium* forty-five minutes old on hanging block.

FIG. 3. Periphery of nineteen hour old culture on agar cone. Relatively heavy inoculum and limited oxygen supply.

FIG. 4. Same cells four hours later (age twenty-three hours).

FIG. 5. Same cells nine and a half hours later (age twenty-eight and a half hours).

FIG. 6. Chain of globoid cells in hanging block culture which had been held under partial anaerobic conditions.

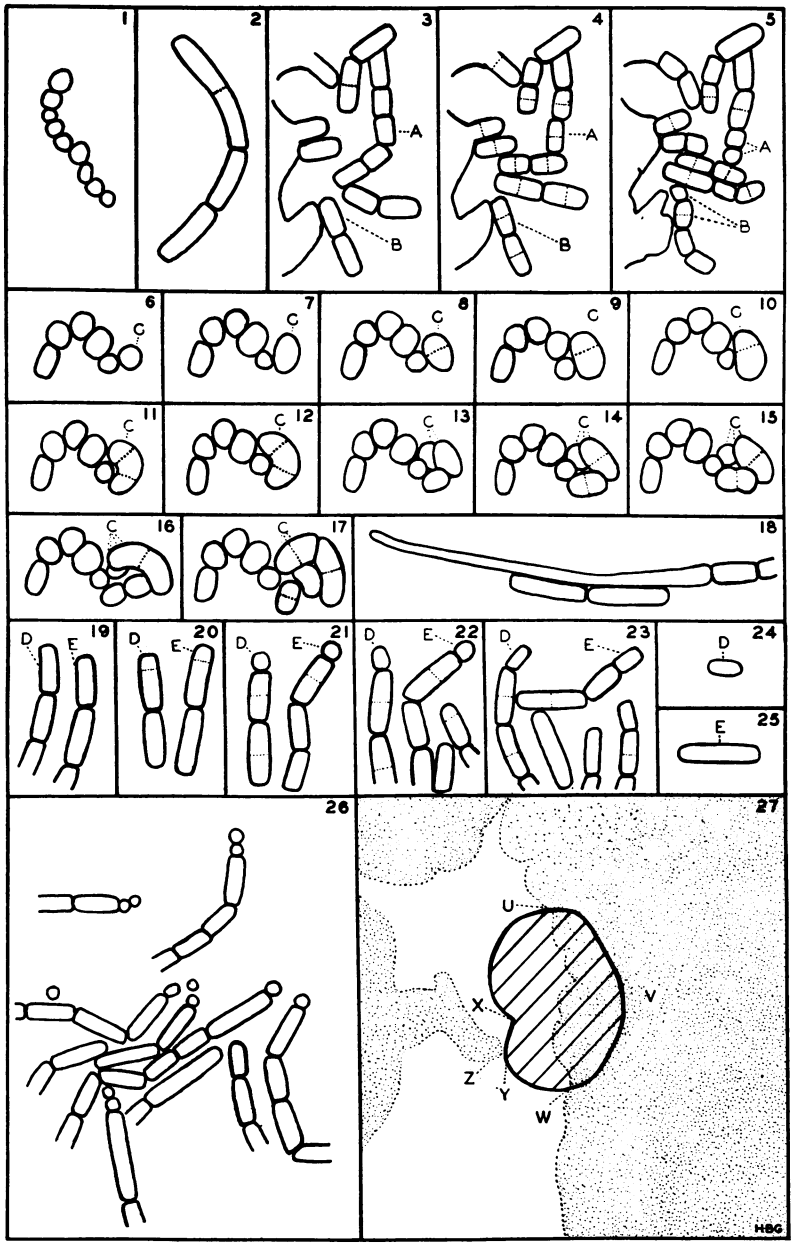
FIGS. 7–17. Same after lower coverglass had been removed to admit air. Drawings made at approximately thirty-minute intervals.

FIG. 18. Runner type cell at periphery of culture three and a half hours old.

FIGS. 19–25. The formation and further development of "round end balls."

FIG. 26. Periphery of twenty and a quarter hour culture showing "round end balls."

FIG. 27. Diagrammatic representation of overlying colony (diagonally lined) and underlying growth (stippled) on a square centimeter hanging block of agar.



(Leo F. Rettger and Hazel B. Gillespie: Bacterial variation)

PLATE 2

Microphotographs of cells growing on hanging blocks of agar

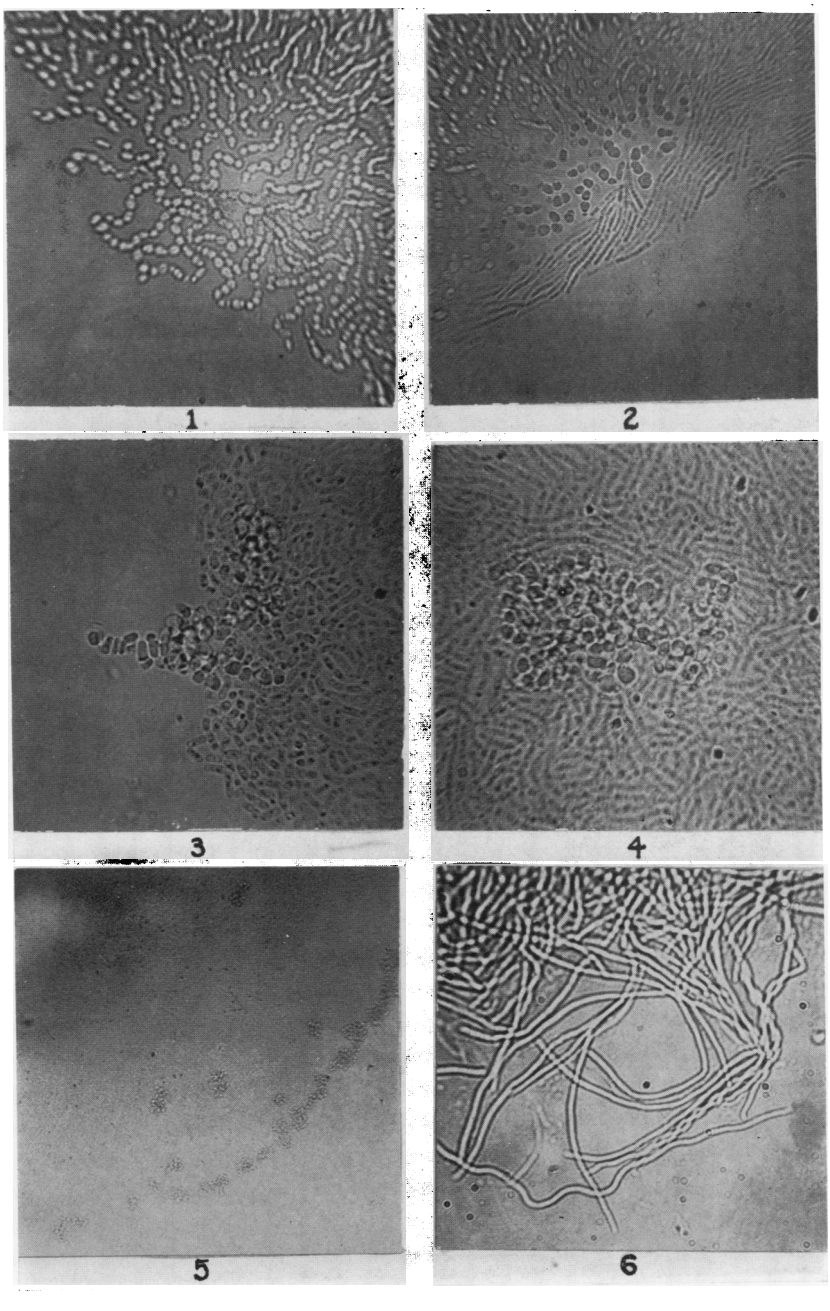
FIG. 1. Globular cells at the periphery of a colony developing under conditions of partial oxygen starvation. ×1000.

FIG. 2. Globular cells developing in a mass of dead and granular "normal" rod forms. ×1000.

FIGS. 3 and 4. Clusters of globular cells lying in masses of dead and autolyzing rods. ×1000.

FIG. 5. Periphery of colony similar to that in figures 3 and 4; shows further autolysis and gives appearance resembling Löhnis' symplasm with round regenerative bodies. ×80.

FIG. 6. Twisting filaments at periphery of a colony. ×1000.



(Leo F. Rettger and Hazel B. Gillespie: Bacterial variation)