SOME NEW OBSERVATIONS ON L FORMS OF BACTERIA1, 2

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The first part of the paper contains observations on the development of the L forms of *Proteus* on the surface of liquid media and the properties of the cultures so obtained. This type of culture was described by Tulasne (1950). These cultures provide L forms in masses which can be separated easily from the media. Our interest was attracted especially by the observation that these cultures differ in important properties from those grown on solid media. Thus, not only do the L forms isolated from various strains often differ considerably, but also L cultures isolated from the same strain differ considerably, and they may be more or less widely separated in their properties from the parent bacteria. In the second part of the paper is described an example of the close connection between the general variability of the bacteria and the production of L forms and the retransformation of these into the usual bacteria. It is also of interest that it was possible with the bacterium described to observe the process by which the bacteria are reproduced in the L forms.

SURFACE CULTURE OF PROTEUS L ON LIQUID MEDIA

Of the L forms isolated from bacteria, only one, the L1 of *Streptobacillus moniliformis*, will grow as a rule when transferred to broth (Dienes and Weinberger, 1951). The L forms of *Bacteroides* and *Salmonella* will grow only occasionally and only if the broth contains a small amount of agar (0.1 to 0.2 per cent). In our laboratory the L forms of other bacteria such as *Proteus* and the gram positive bacteria have not been induced to grow in broth. Growth is not prevented by the lack of nutrients. Colonies will continue to grow on a piece of agar submerged in broth, and sometimes abundant growth develops on heavily inoculated cellophane or cotton which is covered with broth. The physical properties of liquid media are apparently inappropriate. Recently, Tulasne (1950) observed the growth of L forms of *Proteus* on the surface of broth as a heavy film similar to the surface culture of tubercle bacilli. After considerable experimentation, similar growth was obtained in our laboratory from two *Proteus* strains. The influence of physical factors on these cultures is apparent also. They grow rapidly and abundantly on the surface while there is no growth within the medium and only a very slight growth if the inoculum sinks to the bottom.

One of the strains from which a surface culture was obtained is *Proteus* 52. This strain was isolated in 1946 and in the presence of penicillin on serum agar plates produced an abundant growth of the two types of L colonies which were designated as 3A and 3B (Dienes and Weinberger, 1951). The morphology of the organisms is similar in these colonies. They are distinguished by their growth requirements, by the rapidity of growth, and by the tendency to resume the usual bacterial forms. The 3B colonies grow on nutrient agar without animal serum. They grow to a large size (1 to 3 mm) and when transferred to media without penicillin resume immediately the bacterial form. One can observe under the microscope the sprouting and fractionation of the large bodies from which the bacteria are reproduced. The 3A colonies require animal serum and soft agar for growth. When penicillin is eliminated, the bacteria are not reproduced immediately but only after one or several days of growth and so rarely that the mechanism of their derivation has not been observed (Dienes, 1949).

Surface growth in liquid media was obtained only from cultures of 3B colonies. Success sometimes was obtained by partially covering a block of 3B colonies on agar with broth containing penicillin. Positive results were obtained more often in the following way: Melted nutrient agar
containing about 2,000 units of penicillin per ml was inoculated with Proteus strain 52, and slants were made in test tubes. The 3B colonies were well developed in a few days, at which time the slants were partly covered with serum-broth-containing penicillin. The surface growth starts as a thin film which in one or two days covers the surface of the liquid. Transfers "floated" on the surface of fresh media grow rapidly. In two days the culture may cover the surface of broth in an Erlenmeyer flask.

The floating growth develops only in some of the tubes. Using strain 52, of six tubes usually one or two were positive. The results were always negative with fifteen other Proteus strains, three of which were used formerly in the laboratory and twelve others which were freshly isolated from urine and wound specimens. Several trials were made with each strain. All these strains produced 3A and 3B colonies on serum agar. The behavior of various bacterial strains belonging to a species is noticeably different in the production and development of L forms.

The morphology of the floating surface cultures is similar to that of agar cultures. However, in a fresh floating film the organisms are more resistant to distortion and their form is more clearly visible in the preparations. This property allowed the making of successful preparations for electron microscopy. Some of the micrographs are published in the following paper. The floating cultures are similar in some respects to the 3B cultures from which they derive. They grow rapidly and the cultures develop well in nutrient broth without the addition of blood serum. The nutritional requirements of the floating cultures, though less complex than the 3A cultures, are more complex than those of the bacilli. They do not grow in a medium containing ammonium salts, glucose, and nicotinic acid, whereas this medium permits growth of bacilli. Sterile urine is an excellent medium for the floating cultures. Although derived originally from 3B colonies, when the surface culture is transferred to serum agar, the development and appearance of the cultures are similar to that of abundantly growing 3A cultures. In contrast to 3A, small colonies develop from the floating cultures also on nutrient agar without serum and can be propagated on such media for a few generations. The floating cultures differ from the 3B and also from the 3A in the loss of their ability to return to the bacterial form. After a few transfers in the presence of penicillin, the bacillary form of Proteus never reappeared in the cultures. Both 3B and 3A colonies retain unchanged for many months the ability to resume bacterial form.

There was also a difference between the 3A and the floating cultures in their serological properties. The floating cultures gave agglutination to the full titer with antisera produced with Proteus strain 52; the 3A cultures obtained from the same strain were not agglutinated by bacillary antisera (Dienes and Weinberger, 1951). Antisera obtained with two other Proteus strains did not agglutinate this floating L culture. These various observations indicate that the development of floating cultures involves a more profound change in the organism than the adaptation to grow in a changed environment. This culture should be considered a new variant of the L forms differing in some essential properties both from 3A and 3B cultures.

One other floating growth was obtained from an L culture isolated a few years previously from an old Proteus XK strain. This Proteus strain is quite resistant to penicillin; it forms bacterial colonies on plates containing several hundred units of penicillin per ml, but a few small L colonies develop between the bacterial colonies. Cultures were obtained with some difficulty from these tiny L colonies, but after a few transfers they grew abundantly. They were stable from the beginning and the bacilli never could be regained from them. The floating culture of this L form grows slowly. It covers the surface of broth in a test tube in one or more weeks. The type of L form isolated from the XK strain does not conform to any of the types of L cultures isolated from strain 52 or other Proteus strains. It is possible that the small L colonies developing in penicillin plates between the bacterial colonies correspond to 3B colonies, but their ability for growth is restricted. The growth obtained from these colonies on transplants corresponds in properties more to the floating growth than to the 3A type. The culture develops on agar only in the presence of serum. The floating growth develops in nutrient broth or urine without blood serum.

Observations with bacterium B

The Bacterium was isolated in the L form from the sputum of a severe asthmatic patient. The specimen was streaked on ascitic agar plates, and about 2,000 units of penicillin were deposited in a trough cut into the agar in a heavily inoculated
area. The plates were incubated anaerobically. Colonies of the pleuropneumonia-like organisms usually present in the mouth were absent on the area inhibited by penicillin, but much larger colonies were present, similar to the L colonies of bacteria. These colonies grew in similar form when transferred to ascitic agar, but if penicillin were not present, bacilli started to grow in almost all colonies after two or three days' incubation. Observation of the cultures in stained agar preparations at the time when the bacilli were first noticeable disclosed the origin of the bacilli. They developed inside of the large bodies as was formerly observed in *Streptobacillus moniliformis* (Dienes, 1943) and *Escherichia coli*. In these two species the large bodies in which the bacilli developed were produced by the swelling of bacillary forms and not of L forms. In the case of Bacterium B, the large bodies in which the bacilli developed originated from the L form. The bacilli developed not only on the surface of the colonies but also in large bodies situated inside the agar. This strengthens the evidence that the bacilli were derived from the L forms and were not carried as contaminants in the cultures. It is unfortunate that no photographs were made from the colonies during the short period in which the origin of the bacilli could be observed. The edge of the extension of the colony on the surface consisted of a single layer of large bodies. The bacilli were clearly visible in some of these and continued to grow as isolated groups after the disappearance of the membrane of the large bodies.

The bacilli reappeared only in those L cultures which were obtained during a period of a few weeks after isolation of Bacterium B. Cultures of the bacterium were preserved in a CO₂ ice box. The preserved culture stopped spontaneous production of L forms but continued to do so in the presence of penicillin. The L cultures so obtained were stable immediately after isolation, and the bacilli could not be regained from them. The variable morphology of the bacillus, which will be discussed later, remained unchanged during preservation.

The bacilli growing from the L cultures were very small (see photograph 6), about the size of *Brucella*. The cultures usually grew in this form, but occasionally, without apparent reason, their morphology changed in transplants. Only a rare bacterium produced a colony which grew slowly and assumed an actinomyces-like morphology. The young colonies, as is apparent in photographs 2 and 4, consisted of long branching filaments growing into the medium. During the following day, the filaments broke up into bacilli and the organisms slowly resumed the usual small bacterial form. Another transformation was observed more rarely: The small bacilli would start to multiply, but when the colonies consisted of a few dozen bacilli, all the organisms would swell to large bodies from which L type colonies grew. In the fully developed culture only L colonies were present. Bacilli reappeared in these after a few more days. These transformations were observed in stained agar preparations. Direct observation under the microscope was not possible because the time of the transformation was unpredictable. It was apparent in the preparations that the small bacillary forms did not grow as a separate strain in the actinomyces-like or L colonies but that they were reproduced from the bacterial filaments and from the large bodies, respectively.

The classification of this bacterium is uncertain. It is gram negative, both in the small bacillary and in the actinomyces-like form. It is non-motile. It grows only anaerobically in the presence of ascitic fluid or horse serum. Growth could not be obtained in liquid medium. It is unlikely that this organism is an *Actinomyces*. Transitory branching occurs in many true bacteria, especially when they are reproduced from L forms. Similar small bacilli can be obtained from tooth scrapings. They form transitory actinomyces-like colonies and swell into large bodies in the presence of penicillin. However, growth of L forms from these bacilli has not been observed.

These observations are of interest in several respects. One is that the spontaneous production of L forms was connected with a marked variability of the organism in other directions. Immediately after isolation, the transition from bacillary into L forms and vice versa occurred spontaneously. A similar connection between variability and L forms has been observed also in other bacteria, and the spontaneous transition between L₁ and bacilli and vice versa has been observed in certain strains of *Streptobacillus moniliformis*. The characteristics of the L forms of Bacterium B are also of interest. The colonies corresponded in their appearance and in their rate of growth to the 3A type. The colonies multiplied in transplants in the L form, and the bacilli started to develop only in fully grown L colonies and only in a few foci. The 3B type, on
the other hand, in the absence of penicillin immediately reverts to bacteria. In those species in which the appearance of bacilli in 3A cultures was observed, the reversion occurs so rarely that the actual transformation of the organism has not been observed. The example of Bacterium B indicates that the retransformation can occur at a much higher rate in cultures of the 3A type than was observed formerly and that it occurs in a similar way as in the 3B colonies by the development of bacteria in large bodies. It is possible that the 3A forms, immediately after they are derived from bacteria, possess a much greater ability to resume bacterial form than we ordinarily observe in cultures. This ability decreases and usually is lost during long cultivation. The colonies in the original cultures develop slowly and usually require several transfers before they will grow abundantly. During this time their ability to return into bacterial form may be diminished greatly. The observations with Bacterium B are somewhat different from those made with other bacteria, because in Bacterium B the ability of freshly isolated L strains to resume bacterial forms decreased within a few weeks after isolation of the bacterium. This might be compared to the ability to produce viable L forms which usually has been observed to be diminished in old laboratory strains.

GENERAL DISCUSSION

The observations described with Proteus indicate that cultures derived from the same strain and presenting the characteristic morphology of L forms may vary considerably in their properties. Three types of L cultures have been obtained from Proteus strain 52: The 3A and the 3B types formerly described and the floating cultures. Under appropriate conditions these types were stable for long periods of time although the ability to return to bacterial form diminished in all. The 3B type is least different in its properties from the bacterial form. It grows relatively fast, it does not require animal serum, and if penicillin is eliminated it returns immediately to the bacillary form. This type has been observed in Proteus and Salmonella only in the presence of penicillin. In Haemophilus influenzae it is produced also by high concentrations of several amino acids (Dienes and Weinberger, 1951). This type in Proteus will change readily to the 3A form if it is planted on the surface of penicillin-horse-serum-agar plates. The 3A form grows more slowly and has more exacting growth requirements. Its tendency to resume bacterial form is diminished greatly. The 3B form occasionally will transform into floating cultures. This type has similarly low growth requirements and grows as fast as the 3B, but its ability to return into bacilli is soon lost. Reversion of the 3A forms and floating cultures into the 3B form has not been observed.

Almost all strains of Proteus will produce 3A and 3B L type colonies, but their tendency to produce one or the other varies considerably. Of twelve freshly isolated strains, none produced floating cultures. Floating cultures were obtained from one of four Proteus strains isolated a few years previously and from an old Proteus XK strain. The behavior of the last strain was different from that of strain 52 in many respects. It never produced typical 3B colonies and the L colonies were stable immediately after isolation. The floating culture was isolated from these L colonies.

It is apparent that the transformation of the bacterial morphology which characterizes the L cultures does not produce a single variant but a group of more or less closely related forms, and the conditions in the culture determine which will survive. In studying L forms, particularly as to their biological significance, it is important to keep this diversity in mind. It is possible that the forms which fulfill a biological function have not yet been cultivated.

Variation in the properties of the L forms was apparent in Bacterium B also. The L forms produced immediately after isolation of the bacillus were intermediary between the 3A and the 3B types in respect to their ability to resume bacterial form. The bacilli were reproduced from the large bodies as in the 3B L type cultures. The 3A and the 3B types apparently represent a more or less advanced change of the bacterium in the same direction. The cause and function of this change are unknown, and the information we possess does not permit us to make even reasonable suppositions concerning them.

It is of interest that Bacterium B passed through two series of transformations when transplanted in the small bacillary form. In both cases a large part of the transplanted bacteria died and only a few produced colonies. In one series of transformations, an actinomycyes-like growth was observed as a phase between periods of growth in the small bacillary form; in the other series, swelling to large bodies and growth in the L form.
Do these changes serve a similar function, and is it a coincidence that in both cases the intermediary forms were able to grow into the medium? These observations are very complex and without more information cannot be arranged in a simple system.

SUMMARY

Surface growth of L forms in liquid media as described by Tulasne has been obtained from two Proteus strains. With fifteen other strains, such growth was not obtained. The floating growth is similar in many respects to the 3B cultures, but its ability to return to bacterial form is diminished greatly or lost entirely. The morphology of the three different L type cultures obtained from Proteus, the 3A, 3B types, and the floating culture is similar, but they differ considerably in their nutritional requirements, in their rate of growth, and in their tendency to resume bacterial form.

A bacterium isolated from a sputum presented remarkable variability. It grew like a small bacillus, or like an actinomyces, or in the L form. The L form corresponded to the 3A type except that for a certain period after the isolation of the bacterium, the bacilli reappeared in many large bodies in the L colonies. In this respect the L culture was intermediary between the 3A and the 3B types.

The L forms do not represent a single well characterized variant but a group of variants characterized by common morphology, varying considerably in their differences from the parent bacteria.

REFERENCES

Plate I

Figure 1. Floating culture of *Proteus* L covering the surface of broth in an Erlenmeyer flask. One-third natural size.

Figure 2. Young colonies of actinomyces-like growth of Bacillus B. The colonies extend into the medium and they are flattened by drying of a stained agar preparation on the coverslip. × 900.

Figure 3. Surface of a one day old colony of the actinomyces-like growth. Wet stained agar preparation, × 900.

Figure 4. Young growth showing round bodies at the branchings of the filaments. × 900.

Figure 5. Two small "smooth" colonies consisting of tiny bacilli. × 900.

Figure 6. Bacilli in a gram stained smear of "smooth" colonies. × 2,000.

Figure 7. Colonies of the L forms of Bacillus B. Wet stained agar preparation, × 100.