L FORMS ISOLATED FROM A STRAIN OF *SERRATIA*[1]

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ABSTRACT

BANDUR, BOJANA M. (Massachusetts General Hospital, Boston) AND LOUIS DIENES. L forms isolated from a strain of *Serratia*. J. Bacteriol. **86**:829–836. 1963.—In an L culture isolated from a strain of *Serratia*, two types of colonies developed, one (S-1) similar to the usual L colonies with the centers embedded in the agar and the other (S-2) similar in gross appearance to the usual bacterial colonies. The S-2 colonies consisted almost exclusively of large bodies and were produced by the direct multiplication of the large bodies. This occurred by enlargement, deformation, and segmentation. The organisms in the two types of L colonies were equivalent, and the type of growth was determined by environmental influences. The high viability of the cultures and the relative lack of autolysis permitted the study of the reproductive processes and the resulting growth under varying conditions. The basic reproductive process, as in other L forms and pleuropneumonia-like organisms, seemed to be the multiplication of small granules, either free or in small or large aggregates enclosed in a common envelope. It was possible to observe clearly the growth of small granules from the large bodies.

Opportunity for the observations presented in this paper was offered by the development of large L-type colonies on a nutrient agar plate, containing 3% NaCl, 10% horse serum, and penicillin, accidentally inoculated with a strain of *Serratia*. Transfers made from these colonies grew abundantly at 30 C, and growth continued for several weeks until isolated L-type colonies grew to the size of the usual bacterial colonies of this strain. Autolysis was not prevalent in these cultures. Two types of L colonies developed: one type (S-1) corresponded to the usual L colony with a dense center that consisted of small-sized organisms embedded in the agar (Fig. 1). The second type (S-2) had no center embedded in the agar. When examined under low power, the S-2 L colonies had the appearance of the usual bacterial colonies of *Serratia* (Fig. 2) but no bacterial forms were present, and they consisted of large bodies with an admixture of a few small forms like the surface growth of the S-1 L colonies. The S-2 L colonies grew at all concentrations of penicillin tested. Transplants made from both S-1 and S-2 L colonies grew in a similar manner, and the eventual development of one or the other of these two growth patterns appeared to depend to a large extent on the method of inoculation. S-1 L colonies with their agar-embedded centers developed predominantly in lightly inoculated areas of the agar; S-2 L colonies developed in more heavily inoculated areas. A slight growth occurred on transfer of the L colonies to broth with 0.5% NaCl, and more abundant growth resulted from increased concentrations of salt. This growth consisted of large bodies, most of which appeared to be autolyzed and empty, again with a slight admixture of small organisms. The reappearance of bacterial forms has not been observed in the absence of penicillin, either in broth or on agar, from either of these two types of growth.

In the present work we were interested especially in the reproductive processes responsible for the development of the two types of colonies on agar and for the growth in broth.

MATERIALS AND METHODS

The observations described in this paper were made with one strain of *Serratia*. This strain has been used in our laboratory for the absorption of

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oxygen in the preparation of Fortner plates, and its origin is unknown. It grows abundantly, and the cultures grown at room temperature are intensely pigmented. Classification within the *Serratia* was not attempted, because the tendency to produce L forms usually is the property of a bacterial strain. It is not the property of a species or genus. Attempts made a few years ago to induce L-type growth by similar techniques from another strain of *Serratia* were not successful. Two strains obtained from the American Type Culture Collection were highly resistant to penicillin, but they produced large bodies from which L-type growth started. However, this growth remained slight, and L forms did not continue to grow on subculture.

The development of the culture was observed in stained agar preparations made at frequent intervals, and, whenever possible, by observation of the growth in slide preparations with phase contrast. The technique of stained agar preparations has been described several times (Dienes, 1939; Madoff, 1960). The great advantage of this method is that the growth developing both on the surface and within the medium can be studied and photographed.

Trypticase Soy Agar and Broth (BBL) were used in these experiments. The nutritional requirements of these L forms are not high, and other nutrient agars would probably serve as well. Media were tested with varying concentrations of nutrients, agar, horse serum, NaCl, and penicillin.

RESULTS

L forms developed from the bacteria on agar plates that contained no animal serum, but we never observed their development from the bacteria without increased salt concentration of the medium. Transplants of L forms grew best on media containing horse serum and 2 to 3% NaCl. On plates containing 1% NaCl, a slight growth developed with or without horse serum. A few colonies developed, after prolonged incubation, on subculture with 0.5% NaCl and 10% horse serum. Growth was noticeably better when horse blood replaced the horse serum, and the L cultures could be propagated indefinitely on these plates.

The concentration of salt influenced the abundance as well as the speed of growth of the L forms. With 2 to 3% NaCl, growth was detectable after 3 to 4 hr at room temperature. With 0.5 to 1%, only a few large bodies produced progeny, and growth was noticeable only after a few days. The few L colonies (S-1) that developed in these cultures increased in size for several weeks until they reached the size of average colonies grown in the presence of greater concentrations of salt.

The development of L forms from the bacteria followed the usual pattern. The bacilli grew into long filaments in the presence of penicillin. This occurred even with the highest concentration of penicillin tested (3000 to 5000 units per ml). Most of these filaments underwent autolysis and disintegrated; some, however, grew into large bodies up to 20 to 30 μ in diameter. Examination of young cultures in stained agar preparations revealed that the L growth started from these large bodies. Occasionally, after inoculation of the bacilli into the agar, small granules appeared to grow directly from disintegrated bacterial filaments. After a few days of incubation, the bacteria were no longer viable and on subculture only L forms grew.

When transplants of the L forms were made to agar from either broth or agar cultures and examined immediately, large bodies were predominant but were mixed with a few smaller forms and granules. Figures 3 and 5 show the surface of the agar inoculated from agar and from broth, respectively. In slide cultures made from such transplants and studied with phase contrast, only the growth of large bodies was observed. Figures 6 and 8 show the inoculum in a slide culture where the large bodies were isolated and started to develop into S-1 colonies after a few hours of incubation; Fig. 7 shows the same colonies after 12 hr of incubation.

The transferred large bodies were round or slightly polygonal (Fig. 5). After a few hours, the large bodies lying isolated on the surface of the agar became even larger, their peripheries became irregular (Fig. 10), and small granules could be seen attached to their peripheries (Fig. 6 and 8). When examined after 12 hr, the L colonies were visible only at the sites of former large bodies. These colonies consisted of granules of variable size embedded in the agar. On examination of a 24- to 48-hr agar culture, it was found that almost all of the large bodies spread out on the surface of the agar produced colonies.

There were organisms of all sizes in both broth and agar cultures. In transplants made from young cultures, small organisms were not numerous, and we did not attempt to determine the

size of the smallest organism that would grow. When we crushed colonies, embedded in the agar, that consisted almost entirely of small organisms and reinoculated them into the agar, only a few colonies developed.

The tridimensional structure of L-type colonies makes it impossible to obtain clear photographs of the young colonies with phase contrast or to observe the multiplication of single granules. The type of growth originating from the large bodies and producing the young S-1 L colonies is more clearly seen in photographs made from dried stained agar preparations in which the colonies are vertically compressed. Figures 11, 12, and 13 show the earliest stage of the growth of the granules. The newly formed granules embedding themselves in the agar stain darker than the large body and are clearly apparent. In Fig. 13, the granules start to grow at multiple sites at the periphery of the large body as well as beneath it. The large body is deformed and seems to consist of granules also. In Fig. 12, growth starts at four places from the periphery of a large body of moderate size, and two pairs of very small granules are visible (a). Figure 11 shows the growth starting from three large bodies. One (marked a) is the same as in Fig. 12 but it is slightly out of focus. On the second (marked b), also visible in Fig. 12, growth has started at one site and the small granules have produced a darkly stained tiny colony. In the third (marked c), a slight growth is apparent on both sides of the large body. Growth starting beneath the large body is further developed and extends as a broken or twisted filament in the agar. As it is situated inside the agar, this arrangement of the growth cannot be the result of distortion.

A more advanced stage of the development of the colonies is shown in Fig. 14 through 17. The small elongated and double granules are best seen in Fig. 14. To show the granules more clearly, this photograph is reproduced with higher magnification (Fig. 17, $\times$ 3500). In Fig. 16, it is apparent that the smallest granules are at the extending periphery of the small colonies.

The development of the S-2 L colonies followed a different pattern. In this case also, the large bodies increased in size, but, instead of small granules appearing around them and becoming embedded in the agar, their peripheries became irregular with bulges and fissures. At this stage, the large bodies appeared in stained agar preparations to be aggregations of irregular forms 1 to 2 μ in diameter. Figure 9 shows such deformed large bodies. Our observations do not indicate whether the medium-sized organisms, derived from the breaking up of these large bodies, multiply in this form or not. At the periphery of large S-2 L colonies, the medium-sized forms sometimes occurred in masses, many having oblong or irregular shapes. This suggested direct multiplication. On transplant, however, they seemed to swell to large bodies from which further reproduction started.

In broth, mucoid masses were produced which appeared as swirls when the medium was agitated. Multiplication seemed to occur in a manner similar to that of the S-2 colonies but with some modification. This growth in broth consisted mainly of large bodies, many of which were empty and are barely visible in the photograph (Fig. 5). The darkly stained bodies were of various sizes. Again, there was no indication that the smaller forms multiplied directly or grew out from the large forms as in the S-1 L colonies. They were seen free or within autolyzed and otherwise empty large bodies, where they occasionally showed Brownian movement. The arrangement of the stained organisms in the cultures indicated that they were formed and grew to a certain size inside the large bodies.

The main difference between the multiplication in S-2 colonies on agar and the multiplication in broth is that in broth the large bodies remained spherical and did not break up into smaller forms. In both cases, the organisms seemed to develop within the large bodies, in contrast to initiation of S-1 growth on agar, where small granules appeared to grow out of the large bodies and continued to grow in granular form. In solidified gelatin, growth developed in the same way as in broth; the large bodies increased markedly in size but retained their spherical form. Agitation is known to favor the development of L cultures in broth, probably as a result of disintegration of the mucoid masses and breaking up of the large bodies. We have not studied in detail the effect of agitation on the morphology of broth cultures.

The development of the cultures and the morphology of the organisms was markedly influenced by the concentration of agar in an otherwise appropriate medium. On a soft medium, with 0.75% agar and 2% NaCl, only S-1 colonies developed, but the organisms growing into the agar were much larger than with 1.2 to 1.5% agar

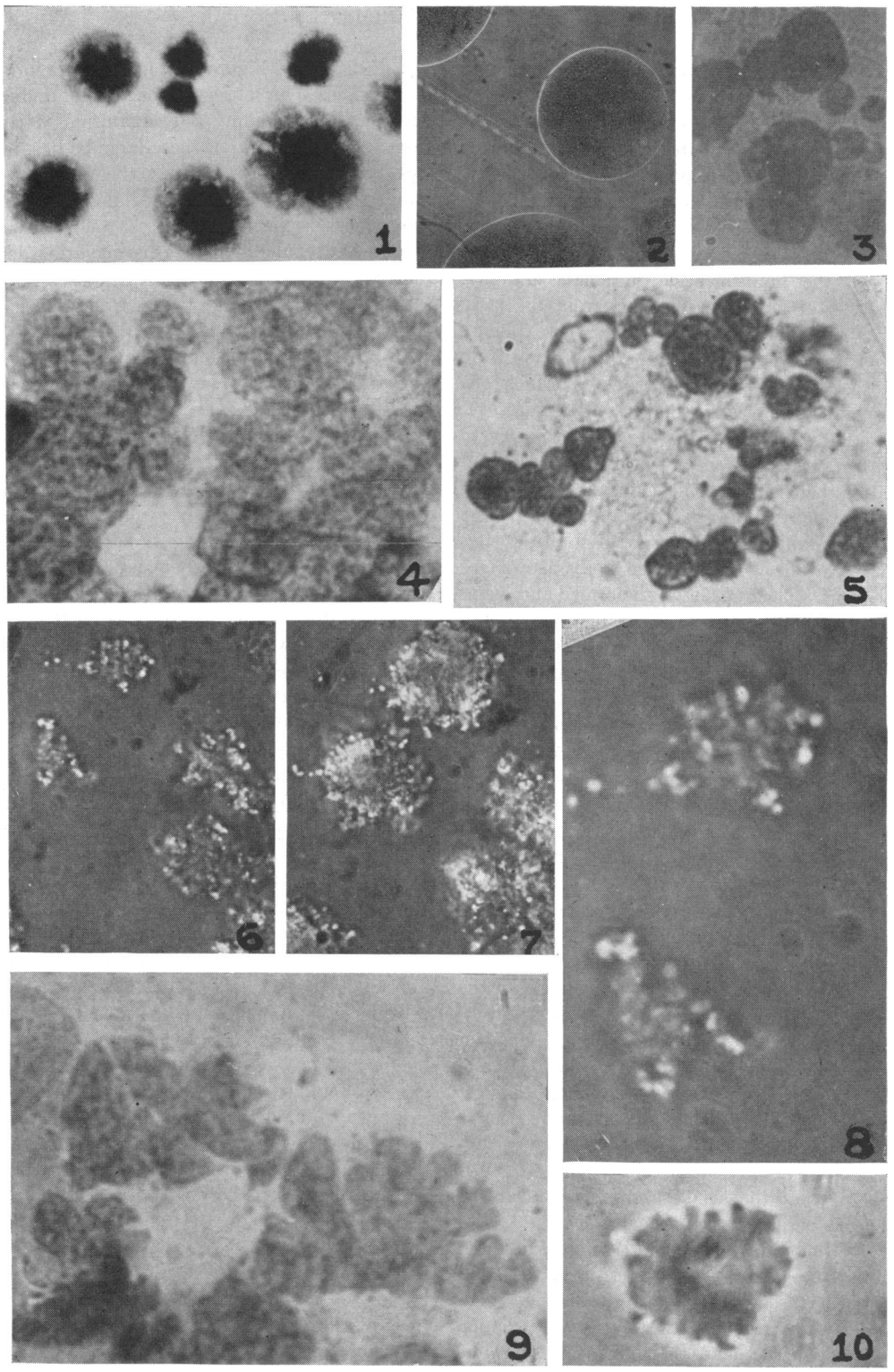

FIG. 1. *L-type colonies (S-1) with embedded centers; 24 hr; × 100. Wet stained agar preparation.*
FIG. 2. *L colonies without embedded centers (S-2). Unstained; × 50.*
FIG. 3. *Large bodies from agar cultures transferred to the surface of another agar plate; × 900.*

and, after 24 hr of incubation, the granules inside the agar grew to 2 to 5 μ in size. On hard medium, with 2 to 3% agar, the growth was noticeably inhibited at first, and only S-2 type colonies developed. These remained small and the large bodies, especially at the periphery, were very large (10 to 20 μ). After 24 hr of incubation, secondary colonies consisting of very small granules developed under the large bodies. This secondary growth continued to increase for several weeks, and at the edge of the inoculated area thick, opaque colonies developed which had irregular peripheries. Autolysis was not prevalent in these colonies. The granules in old colonies increased in size, but no large bodies developed on the surface or periphery of the colonies.

Not only the various processes of multiplication but also the appearance of the large bodies suggest that at a certain stage of their development they consist of small granules similar to those which grow out of them. This was visible with phase contrast at the same time that the granules started to grow out of them. The granules in the large bodies are visible in stained preparation (Fig. 4), and their enlargement before the segmentation of the large bodies can be seen.

Discussion

The L form isolated from *Serratia* attracted our interest primarily because of the development of S-2 colonies which seemed to be produced by the direct multiplication of the large bodies. The large bodies which develop in bacterial cultures, in L forms, or in pleuropneumonia-like organisms (PPLO) are usually not observed to multiply in this form. If they reproduce, they produce bacteria or the small granules of L forms or of PPLO. The origin of the large bodies at the periphery of L colonies of *Proteus* was carefully studied (Leibermeister, 1960). Multiplication seemed to occur only in the embedded center of the colonies. The periphery of the colonies on the surface of the agar is apparently produced by the extrusion of the organisms from the center and their enlargement into large bodies. A similar origin of the large bodies at the periphery of the colonies is suggested in the L forms of the pneumococcus (Madoff and Dienes, 1958). One instance of direct multiplication of the large bodies was observed by Tulasne and Lavillaureix (1957) in an old L culture of *Vibrio cholerae*. The multiplication of the large bodies in the L forms of *Serratia* could be observed in the S-2 colonies and at the periphery of the S-1 colonies. This process followed the pattern observed in a strain of *Bacteroides* when bacteria were reproduced from the large bodies (Dienes and Smith, 1944). The large bodies increased in size, became irregular in outline, and broke up into irregular segments. In the case of *Bacteroides*, this occurred only in large bodies produced directly from bacteria and in the absence of penicillin or other inhibitory substances. The fragments of the large bodies resumed the usual bacterial structure during further development. The L forms of *Serratia* lost the ability to return to bacterial growth and the fragments of the large bodies grew again into large bodies, both in the presence and in the absence of penicillin. The similarity of the process observed in *Serratia* to the reproduction of bacteria from the large bodies in other bacterial species suggests that in the L form of *Serratia* the functions of the cell membranes are less altered than in many other L forms. This is sug-

FIG. 4. *Large bodies at the periphery of L colonies. Note granules inside the large bodies. Wet stained agar;* × *2250.*

FIG. 5. *Large bodies from broth, some darkly stained, and some degenerated and unstained. Note granules inside them. Wet stained agar;* × *2250.*

FIG. 6. *Earliest growth of L-type colonies with embedded centers (S-1). Large bodies after 6 hr of incubation at room temperature in slide culture. Phase contrast,* × *900. The small granules growing from the large bodies are bright, and the granules are visible in the large bodies.*

FIG. 7. *Same field as Fig. 6 after 12 hr of incubation. Phase contrast,* × *900.*

FIG. 8. *Detail of Fig. 6, further enlarged;* × *2250. The granules growing out from the large bodies are bright and, where resolution is good, are very small. The boundary of the large bodies is not sharp; they appear as groups of granules.*

FIG. 9. *Earliest growth of the large bodies leading to colonies without embedded centers (S-2); 6 hr after inoculation from agar culture. They are deformed with protuberances breaking into fragments. Larger divisions and granules are visible in the large bodies. Wet stained agar;* × *2250.*

FIG. 10. *Large body after short incubation showing growth starting from the periphery (S-2). Unstained;* × *2250.*

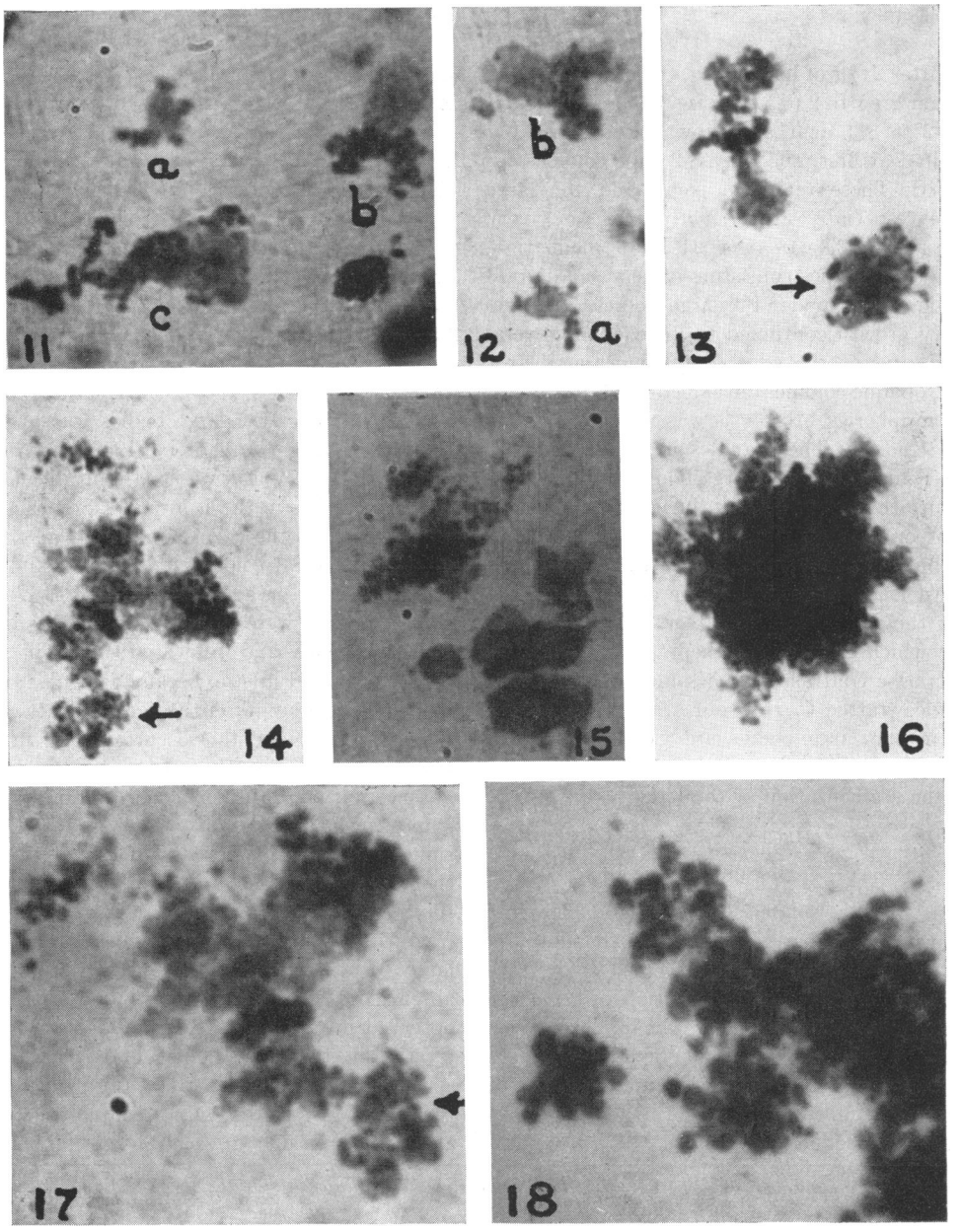

FIG. 11. *Growth of small granules from the large bodies leading to the development of S-1 colonies. Large bodies marked a show the earliest growth of the small granules. (b) Small colony of granules growing from the large body; (c) growth of granules from the large body; at the left, granules arranged in a twisted filament. Dried stained agar preparation;* × *2250.*

FIG. 12. *Same as a and b in Fig. 11, with different focus. (a) Several pairs of small granules are visible growing from the large body.*

FIG. 13. *Large body deformed with granules growing out of it at several sites (arrow). Dried stained agar preparation;* × *2250.*

FIG. 14 and 15. *Extension of very small granules in different directions inside the agar. Dried stained agar preparations;* × *2250.*

FIG. 16. *Large colony extending in different directions inside the agar. The smallest granules are at the periphery. Dried stained agar preparation;* × *2250.*

FIG. 17. *Same as Fig. 14;* × *3500. Enlargement shows the growth of the granules more clearly.*

FIG. 18. *Growth (12 hr) of human PPLO strain (4330). Impression from dried agar preparation;* × *2500.*

gested also by the high viability of the large bodies and the relative absence of autolysis.

The morphology of the *Serratia* L cultures and their method of reproduction are influenced markedly by their environment. Most conspicuous are the effects of the salt concentration and the presence or absence of agar and the concentration of agar in the medium. Whether the organisms are crowded or are widely distributed on the medium exerts a marked influence also. These influences are complex and do not allow for a simple explanation of the different patterns of development in these cultures. Multiplication in the form of small granules was observed only on agar media. In very soft agar (0.2 to 0.4%), the large bodies multiply as they do in broth. With the usual concentration of agar (about 1.2 to 1.5%), small granules grow out from the large bodies lying isolated on the surface of the medium, but growth was initiated only by the large bodies and not by small granules transferred to the agar. In transplants, crowding inhibits the growth of granules from the large bodies. The size of the granules and the initiation of their growth are greatly influenced by the concentration of the agar. High concentrations of agar inhibit the starting of growth of granules from the isolated large bodies and favor the direct multiplication of the large bodies themselves (S-2). However, after some growth, secondary colonies of very small granules grew under the large bodies. A similar growth of granules occurred on soft agar also under old isolated colonies. Probably it is an analogous phenomenon that inoculation of the agar near a well-grown culture produces small colonies consisting of granules, but the colonies develop more abundantly than after inoculation of fresh medium. Thus, the influence of crowding may be either inhibitory or favorable for the growth of granules.

We have observed no signs of multiplication of the small granules in liquid medium. Multiplication in broth seems to correspond to the growth processes of the S-2 colonies. The new organisms are formed and develop to a certain stage inside the large bodies. This process could be followed more clearly in the broth cultures of the L forms of gram-positive cocci and will be discussed elsewhere.

The basic cause of pleomorphism in bacteria, L forms, and PPLO seems to be not a monstrous growth of single organisms but rather the lack of separation of the growing units from one another (Dienes and Smith, 1944; Dienes, 1960). This is apparent in the formation of filaments and chains in bacteria and in some strains of PPLO. In some cases, it was possible to observe that the young large bodies developing from bacteria are analogous structures. Under appropriate conditions, they divide into groups of bacteria preformed in them, like bacterial filaments (Dienes and Smith, 1944). Whether the units capable of individual growth are fully separated or the large body is a multinuclear organism is undecided. In some strains of bacteria, transition between bacterial forms to large bodies occurs as readily as in L forms and depends just as much on the environment (Dienes, 1942). That the large bodies of the L forms of *Serratia* at a certain stage of their development consist of granules of variable size was visible in microscopic preparations. It was also indicated by the growth of small granules from them at multiple sites at their surface.

The growth of small granules from large bodies and their continued development in the early L colonies was more clearly observed in *Serratia* S-1 L colonies than in any other L forms. It is apparent that the granules are well-defined organisms and not leaks of protoplasm between the mesh of the agar gel. One of the most interesting problems in the study of L forms is to determine the conditions that permit their growth. They grow apparently in the large bodies and inside the agar, and substances diffusing from the large bodies and from growing colonies may be both antagonistic and favorable for their growth. Our knowledge of the large bodies and of the granules of the L forms is limited, and the present work must be regarded as preliminary to more penetrating studies.

The size of the smallest granules capable of multiplication has played an important role in the comparison of PPLO and L forms. The marked influence of the environment on the growth of the small granules of the L forms and on their size makes it difficult to make a comparison of any significance. The size and shape of the small granules in young agar colonies of the two groups are closely similar. For the sake of comparison, a photograph of vigorously growing young colonies of PPLO strain 4330 of human origin is shown in Fig. 17. The organisms in these small colonies are considerably larger than those made from comparable colonies of the L forms of *Serratia*.

Two recent publications (Altenbern and Land-

man, 1960; Meloni and Monti-Bragadin, 1962) described the multiplication of the large bodies in such form. Unfortunately, the morphology was not followed throughout the whole development of the cultures, and the possibility remains that the large bodies might have been produced by pleomorphic bacteria. The organisms in certain strains of bacteria after some growth are transformed to large bodies *in toto*, but multiplication occurs in bacterial form. The pleomorphism of bacteria and the growth of L forms are closely related; but, there are differences between the two phenomena, and it is advisable to keep the information relating to them apart.

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LITERATURE CITED

ALTENBERN, R. A., AND O. E. LANDMAN. 1960. Growth of L-forms of *Proteus mirabilis* in liquid media. J. Bacteriol. **79**:510–518.

DIENES, L. 1939. L organisms of Klieneberger and *Streptobacillus moniliformis*. J. Infect. Diseases **65**:24–42.

DIENES, L. 1942. The significance of the large bodies and the development of L type of colonies in bacterial cultures. J. Bacteriol. **44**:37–73.

DIENES, L. 1960. Controversial aspects of the morphology of PPLO. Ann. N.Y. Acad. Sci. **79**:510–518.

DIENES, L., AND W. E. SMITH. 1944. The significance of pleomorphism in Bacteroides strains. J. Bacteriol. **48**:125–153.

LIEBERMEISTER, K. 1960. Morphology of the PPLO and L forms of *Proteus*. Ann. N.Y. Acad. Sci. **79**:326–343.

MADOFF, S. 1960. Isolation and identification of PPLO. Ann. N.Y. Acad. Sci. **79**:383–392.

MADOFF, S., AND L. DIENES. 1958. L forms from pneumococci. J. Bacteriol. **76**:245–250.

MELONI, G. A., AND C. MONTI-BRAGADIN. 1962. Osservazioni sull'isolamente dall'uomo di sferoplasti "spontanei" da un ceppo di *Klebsiella*. Ann. Sclavo **4**:143–152.

TULASNE, R., AND J. LAVILLAUREIX. 1957. Sur une variante geante, homogene et stable issue de bacterie du genre vibrion. Schweiz. Z. Allgem. Pathol. Bakteriol. **20**:608–613.