THE SIGNIFICANCE OF THE LARGE BODIES AND THE DEVELOPMENT OF L TYPE OF COLONIES IN BACTERIAL CULTURES[1]

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The occurrence of deeply stained large round bodies in bacterial cultures has long been known. These large bodies have aroused much speculation, but from the study of the literature nothing definite can be learned concerning their nature and functions, and the majority of bacteriologists regard them as products of degeneration. The author has previously pointed out (1939a) that the large bodies which give the characteristic appearance to the cultures of L_1 and of the pleuropneumonia group of organisms are morphologically very similar to the large bodies of bacteria and are probably analogous in nature. If this supposition is correct, we must obtain more information concerning the nature of the large bodies in order to understand the nature of L_1 and of the pleuropneumonia group of organisms and their relationship to other microorganisms.

The large bodies in most cultures degenerate without further development. It is not unreasonable to expect that in some cultures their functions will be apparent if we study a large number of cultures belonging to different species. With this thought in mind, many routine bacteriological cultures have been examined during the last three years for the occurrence of large bodies. When they were found, their properties and their eventual development was studied both in the original cultures and in transplants. Between 3,000 and 6,000 cultures were examined although their exact number is not known since no records were kept of the cultures which showed no, or only a few, large bodies. By this procedure strains which produced large bodies in abundance were found in many bacterial species. They occurred only occasionally among freshly isolated strains of *Escherichia coli*, *Hemophilus influenzae* and *Hemophilus parainfluenzae*, gram-positive aerobic sporebearing bacilli and *Streptococcus*. Colonies in which nearly all cocci transformed into large bodies were frequently seen in gonococcus cultures grown directly from patients, and all strains of *Streptobacillus moniliformis* and *Bacteroides funduliformis* isolated from pathological lesions produced large bodies abundantly. Photographs of the large bodies in various cultures have been published previously (1939a) and Pictures 1 to 4 in plate 4 show the different stages in the transformation of colon bacilli into large bodies.

The most important result obtained by the study of these strains is the observation of the germination of the large bodies. They usually do not reproduce

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bacteria of regular shape but small granular forms which are similar in morphology and further development to the organisms of the pleuropneumonia group. The best-known instance of the germination and further development of the large bodies is the appearance of L_1 colonies in cultures of *Streptobacillus moniliformis*. The significance of this observation depends largely on whether this pleuropneumonia-like growth is a variant of the bacteria or a symbiont, as Klieneberger supposed. For the study of this problem the *Streptobacillus moniliformis* is most appropriate, and a large part of the observations described in this paper have been made with this organism.

The observations have accumulated slowly during several years and involve a large number of strains. It required every effort to arrange them in an orderly way. They are grouped under the following headings: (1) technical procedures; (2) morphology and origin of the large bodies; (3) the function of the large bodies; (4) reversion of the L_1 into bacillary forms; (5) germination of the large bodies in cultures of L_1; (6) autolysis and variability of *Streptobacillus moniliformis;* (7) L type of growth in cultures of a *Flavobacterium;* (8) L type of growth in colon bacillus cultures.

A part of the observations described in this paper have already been published in short notes (Dienes, 1939b, 1940a and b, 1941a).

1. TECHNICAL PROCEDURES

Before proceeding further it is necessary to describe the technique used. The large bodies developing in bacterial cultures and the L type of growth are very fragile, and in the usual dry smears they are deformed and often cannot be recognized. They are better maintained in impression preparations, especially by using the agar fixation method of Klieneberger (1934). However, even in such preparations the details of their morphology disappear, and the relationship of the different elements of the cultures is distorted. Furthermore, in impression preparations the young L type of growth which is partly embedded in the agar is not visible or is considerably distorted. Direct microscopical examination of unstained cultures is unsatisfactory because it is difficult to recognize the L type of growth in such preparations, and the viable and autolzyed elements of the culture cannot be distinguished. Dark field cannot be used for examination of colonies *in situ* on the surface of the agar, and in liquid media it is usually impossible to follow the derivation of the different elements in the cultures. The observations which will be described later were made possible only by the use of a method outlined in a former paper, (Dienes, 1939a) which allows the staining of the cultures *in situ* on the surface of the agar. In reading the following descriptions it must be kept in mind that the preparations contain with minimum distortion, the whole growth developing on the media, and that the various bacterial structures are clearly stained.

The technique in its simplest form is as follows: An alcoholic solution of methylene blue and azure is spread over the surface of a coverslip and allowed to evaporate. A small square of agar bearing the colonies to be studied is cut out of the plate and transferred to a slide. The previously stained coverslip is then placed down upon the agar, avoiding any sliding motion. The colonies

on the agar take up the stain from the coverslip without any distortion of their elements. Lastly, molten paraffin is run around the agar block and allowed to harden in order to fill the space between the coverslip and the slide. This staining is semivital as the stained bacteria remain motile and viable. During the last year the staining technique has been improved by covering the coverslip, with a loopful of 0.5 per cent phosphoric acid after evaporation of the alcohol. After evaporation of the water a bright blue film remains. The culture comes in contact first with the acid film which exerts a fixative action and produces more uniform and stronger staining than the dyes alone.

The preparations made with this technique keep only for a few days. Furthermore, the L colonies cannot be photographed in these preparations because they grow into the media and cannot be focused together with the surface of the agar. A permanent dry preparation can be made in the following way: A section, about 0.5 to 1 mm. thick, is cut from the surface of the agar block with a razor blade and is placed on the coverslip covered with phosphoric acid, methylene blue and azure. The preparation is kept for two to five minutes in a wet chamber to prevent drying. Then a small piece of filter paper is placed on the agar and the preparation is put in a warm place where it will dry quickly. The filter paper absorbs most of the moisture present in the agar. For this reason the drying is quicker and the stain is not decolorized. Sometimes it is advantageous to wash the agar film by putting a small drop of water on it repeatedly and then to draw it off after a short time by touching the edge of the agar with filter paper. In doing this, water should not get between the agar film and the coverslip as it ruins the preparation. When the agar film is completely dry it is moistened with xylol and mounted in Canada balsam or some other appropriate mounting medium. The dry preparations are not as satisfactory as the wet ones because such unstained elements as autolyzed bacteria and large bodies cannot be seen in them, and the granules and vacuoles of the large bodies remain barely visible. Being compressed vertically, the arrangement of the different elements in the colonies is not as apparent as in wet preparations. For these reasons they cannot take the place of the wet preparations. Their great advantage, besides being permanent, is that the L colonies can be photographed in the dry preparations. Many of the photographs included in this paper were made from such preparations. A great advantage of both the wet and dry preparations is that they can be made very quickly and can be examined simultaneously with the plates from which they are made.

Many variations of the technique were tried to improve further the staining of agar cultures. For certain purposes fixation with $HgCl_2$ has given better results than fixation with phosphoric acid. However, if $HgCl_2$ is used, the procedure is not so simple and the distortion of the colonies cannot be so easily avoided as it can by following the technique described above.

2. MORPHOLOGY AND ORIGIN OF THE LARGE BODIES

In freshly stained agar preparations, the morphological appearance of the large bodies was similar in all cultures studied, including those of *Streptobacillus moniliformis* and of the pleuropneumonia group of organisms. The staining

properties of the granules and vacuoles developing in the protoplasm were similar, and the large bodies disintegrated in the same way by transforming into a bleb filled with liquid, or into a granular mass if the membrane disappeared. It could also be clearly observed that the large bodies developed from the bacteria. It was necessary to return to this subject only because Klieneberger (1940) in a recent publication maintained her opinion that the large bodies in the cultures of the *Streptobacillus moniliformis* are exceptional and cannot be compared to those present in other bacterial cultures.

The details of organization of the large bodies, such as the presence of a membrane and vacuoles and granules, are visible only in stained wet preparations. They disappear or remain barely visible in dry preparations and also in those made with the agar fixation technique. It is interesting to compare photographs 2 and 3 in table IV, one representing the large bodies in dry, the other in wet preparation. The early autolysis of the large bodies in the cultures of L_1 and of the pleuropneumonia group of organisms obscures their similarity to the large bodies of bacteria. In many cases in the pictures published by Klieneberger (1938, 1940) only the foamlike structure produced by autolysis is represented. The autolysis of large bodies produces very similar structures in cultures of certain strains of *H. influenzae* and of the gonococcus. These structures, as Klieneberger (1940) remarked, are very different from swollen bacteria. However, when the large bodies are compared in corresponding stages of development, they are similar in every respect.

It seemed worth while to publish photographs of dry impression preparations of the large bodies stained with Giemsa stain (plate I, photographs 1 and 2). In these such a similarity exists between the large bodies of the pleuropneumonia group of organisms and of the bacteria that is can hardly be accidental. The nucleus-like chromatin granules, such as are seen in Ledingham's (1933) picture of the large bodies of the organism of bovine pleuropneumonia are apparent in these preparations in the large bodies both of L_1 and the colon bacillus. The large bodies of *H. influenzae* and gonococci show similar structures. These nucleus-like structures were indicated in swollen bacterial forms some time ago by Kritchewsky and Ponomareva (1934). The filaments with which these "nucleated" bodies are connected in Ledingham's pictures are most probably artifacts produced by the effects of tearing. They are seen often in distorted colonies but are never present when the colonies are not disturbed.

According to Klieneberger (1940), the large bodies in cultures of *Streptobacillus moniliformis* are not only morphologically different from those of other bacteria, but they do not originate from the bacteria. According to her observations the bacillary colonies always contain nonbacillary elements belonging to a symbiotic L_1. When the colonies are transported, these nonbacillary elements start to multiply independently of the bacteria and the large bodies are produced by them.

Klieneberger regards all pleomorphic bacillary forms and also the large bodies in the bacillary colonies as forms of the symbiotic L_1. For this reason her state-

ment that elements of L_1 are visible in all bacillary colonies means only that besides regular bacilli the colonies contain pleomorphic forms. The supposition that the regular bacilli and the pleomorphic forms have a different origin is not supported by direct evidence. Originally Klieneberger (1935) regarded the so-called "filamentous network" as an evidence for the presence of L_1 in the bacillary colonies. When she recognized (1940) that the network was not a genuine structure, all morphological evidence formerly presented for the symbiosis hypothesis was eliminated. The author cannot agree with Klieneberger's statement that the swollen pleomorphic bacillary forms could be produced by a soft globular body "lying on top or surrounding the bacillus." The gradual swelling of the bacilli is visible in stained wet preparations, and it is sometimes apparent that the swollen forms have a membrane which is a continuation of the bacillary membrane and that their content, including vacuoles and granules, extends into the bacillary filament connected with them. No morphological difference is present between the large bodies of *Streptobacillus moniliformis* and of other bacteria.

As regards the mixed growth of L_1 and the bacillary colonies in the transplants, it is more difficult to compare Klieneberger's and the author's observations because the properties of different strains vary considerably, and the cultures are strongly influenced by the conditions of cultivation. In certain cases in which the author observed a mixed growth, the cultures were grossly mixed and could be easily separated by picking isolated colonies. It will be described later in this paper how under certain conditions L_1 appears regularly in the cultures by the germination of the large bodies. It is possible that a similar process occurred in Klieneberger's cultures.

While certain strains of *Streptobacillus* under appropriate conditions give a mixed growth, many strains can be transferred on ascitic agar plates for long periods and L_1 colonies never appear in young cultures. They develop only in old colonies as a secondary growth a long time after the large bodies are produced. This observation was discussed in a former paper. During the last two years several strains have been studied, and in most cultures no L_1 developed simultaneously with the bacillary forms. When L_1 develops in the cultures it is so conspicuous that it could not have been missed in the preparations.

The observation that the L_1 is not visible in many cultures of *Streptobacillus* is more important than the occurrence of mixed cultures. Mixed growth in itself, gives no information concerning the origin of the large bodies. On the other hand, the development of large bodies in cultures in which the characteristic elements and colonies of L_1 are absent, is definite evidence against the symbiosis hypothesis.

The intensity with which large bodies are produced varies considerably in different strains of *Streptobacillus moniliformis*. In many strains isolated from the nasopharynx of healthy rats the bacterial filaments show no pleomorphism at all, and in pleomorphic strains variant colonies which are not pleomorphic appear after long cultivation. The transformation into large bodies represents

an exceptional condition of the cultures as in other bacterial species. In colonies in which most of the bacteria are involved, the fusiform swelling develops with great regularity in the bacterial filaments as is visible in photograph 5 of plate I. In the strains in which only a few large bodies develop, certain parts of the bacterial filaments are more involved than others. These are often twisted and fragmented. For this reason, when the large bodies are fully developed they are in clusters without visible connection with the bacteria. The examination of the colonies in successive stages of development shows that these clusters of large bodies develop not from the symbiotic growth of L_1 but from bacterial elements. The gradual swelling of bacteria is visible in photographs 3, 4 and 5 of plate I. In photograph 4 the twisting and fragmentation of the filaments and their gradual swelling are apparent. Klieneberger regards such clusters of large bodies in cultures grown in liquid media as evidence for the independent growth of the large bodies and the bacteria.

3. THE FUNCTIONS OF THE LARGE BODIES

The first information as to the function of the large bodies was obtained with an influenza bacillus strain (Dienes, 1939b). A broth culture of this strain contained many large bodies as well as a few bacilli, and after transplanting to a blood agar plate, these large bodies were seen lying singly on the surface of the agar. After eight hours' incubation the bacteria developed into tiny bacterial colonies. A very different growth, however, started from many of the large bodies. This consisted of small granules and diphtheroid-like forms which were much more deeply stained with methylene blue than were the bacteria. This growth, instead of spreading on the surface of the medium like the bacterial colonies, invaded the media beneath the large bodies, producing a structure closely similar to a young L_1 colony. As this growth developed exclusively in connection with the large bodies, and as no elements similar to it were noticeable in the broth culture, it is reasonable to suppose that it started from the large bodies. The fact also that the growth did not develop into a bacillary colony indicates that it did not consist of usual bacilli. This growth remained very small and the author did not succeed in perpetuating it in transplants. The L type of colonies was observed in only one other influenza strain. This was isolated from the blood in a case of acute sepsis.

The germination of large bodies was observed next with colon bacilli. From large bodies lying singly on the surface of the agar the same type of deeply stained granules and small diphtheroid-like forms as was observed with influenza bacilli grew into the agar and produced a tiny colony. These never developed into bacterial colonies and did not grow in transplants. The development of these tiny colonies in cultures of the colon bacillus will be described and illustrated in a following section of this paper.

After the development of the large bodies was observed in these cultures, efforts were made to observe this process in the cultures of *Streptobacillus moniliformis*. The first success was obtained on an alkaline agar plate (pH 8.2) without addition of serum or ascitic fluid. The bacilli grew on these

plates only on thickly inoculated sites. They did not multiply and did not take the stain on thinly inoculated areas. A few of the large bodies remained intact and well stained and the same type of growth started from them as was observed with influenza and colon bacilli. This growth did not develop into visible L_1 colonies and after forty-eight and seventy-two hours only a few granules remained stained in them. The main interest of this observation is that on plates which do not support the growth of L_1 the germination of the large bodies produced a structure similar in every respect to the structures produced in influenza and colon bacillus cultures.

In further experiments it was observed that the large bodies germinated also on plates containing ascitic fluid or blood if they were incubated between 25 and 30°C. For these experiments three different strains of *Streptobacillus* were used. The first was the same used in the experiments described in a former paper (1939a). It produced large bodies fairly abundantly, but these were autolyzed to a large extent after forty-eight hours of cultivation. The second strain was isolated from a lung abscess of a white rat. The bacteria in this strain had a stronger tendency to develop into large bodies than in any other strain, and the large bodies remained intact for a relatively long period. After forty-eight hours a colony apparently consisted only of deeply stained round bodies. The strain was highly virulent for mice. The third strain was isolated from the nasopharynx of a healthy rat. It grew in long regular filaments which produced relatively few large bodies and was only moderately virulent for mice. The properties of these strains remained constant in mouse passage, indicating that even in freshly isolated strains marked individual differences are present.

The observations made with these three strains were similar in all essential points and will be described together. When the strains were propagated on boiled blood ascitic agar plates at 36°C. they grew exclusively in the form of bacterial colonies, among which L colonies never developed. The large bodies transferred with the inoculum were visible on the surface of the agar but they did not show any development and after some time disintegrated. After two to three days' incubation a few L_1 colonies developed beneath the bacterial colonies as a secondary growth. When the plates were inoculated with cultures grown between 25 and 30°C. and incubated at this temperature a different growth developed. On the areas where the agar was thickly seeded the bacilli grew in confluent colonies. In the sparsely inoculated areas many L colonies developed together with a varying number of bacterial colonies. When the plates were examined after eight to twelve hours' incubation, it could be seen that the L colonies developed from the large bodies lying singly or in small groups. The first stage of the germination was very similar to the process observed in colon and influenza bacillus cultures. After incubation overnight the growth starting from the large bodies developed into tiny L_1 colonies, in many of which the large bodies were still visible. The large bodies which gave rise to L colonies often considerably increased in size and contained granules corresponding to the L_1 growth. Occasionally a large body was seen breaking up into granules. The morphological change of the large bodies gives further support to the con-

clusion that the L_1 actually grew from the large bodies and the connection between them was not accidental. The L_1 grows well on this medium, and if it did not originate from the large bodies but from some other elements of the culture, a constant association with the large bodies would not be expected. The direct observation of the germination process under the microscope would not be more conclusive than the stained preparations. The L_1 cultures consist partly of very small elements, and the accidental association of these with the large bodies cannot be definitely excluded by microscopical examination of unstained preparations.

The examination of the photographs will give a fairly accurate impression of the actual observations, though the photographs are less clear than the stained wet preparations. The distinctive bright-blue color of the viable bacterial structures is absent in the photographs and the flattening out makes the structures of the L colonies and their connection with the large bodies less distinct. The pictures were selected mainly to illustrate the germination of the large bodies, but several of them illustrate at the same time the development of the large bodies from the bacilli.

Photograph 3 on plate I shows the edge of a 24-hour culture of Strain 3 grown at 25 to 30°C. The bacterial filaments show various grades of transformation into large bodies from a slight swelling up to a definite fusiform appearance. The transformation is not complete and the large bodies are all connected with bacteria. Such cultures were used for transplants. Photograph 7 shows the development of transplants after 12-hour incubation. Both the large bodies and the bacteria are of interest in this photograph. In the center of the picture are three large bodies from which granules and small globular forms characteristic of L_1 are shown growing out in various directions. Part of this growth is under the surface and it is therefore not in perfect focus. The large bodies themselves are considerably increased in size. The arrangement of bacterial filaments does not show any signs of multiplication. They show fusiform swelling, and at certain places the bacterial filaments are fragmented. By the swelling of these fragments groups of large bodies are produced. This process, which was referred to in the preceding section of the paper, is also illustrated in another photograph (4). Photograph 6, made from the same preparation as 2, shows a large body in the center from which L_1 has started to grow in four directions. The photograph also shows a group of large bodies which did not germinate. In photograph 8 the large body from which L_1 starts to grow is not in sharp focus. Photograph 9 shows a small L_1 colony after 24 hours' incubation between 25 and 30°C. A small colony was selected because the elements composing such colonies and the similarity of these elements to the growth developing from the large bodies are visible. Photographs 1 to 6 on plate II illustrate the development of L_1 colonies in the cultures of Strain 2. In photograph 1 an agar plate is shown with low magnification after incubation overnight at 25 to 30°C. Photographs 2 to 7 show growths on the same or adjacent fields with high magnification. Where the plate was heavily inoculated the large bodies and bacteria show no development, but a large body at the edge of the

inoculated area is considerably increased in size (photograph 2). In the thinly inoculated area tiny L_1 colonies developed. In many of these, the large bodies from which they developed are still visible. Such colonies are illustrated in photographs 3 to 7. The cultures of this strain used for the inoculation of the plate consisted mainly of large bodies. For this reason, in the sparsely inoculated areas only a few bacterial colonies developed. In the thickly inoculated areas the large bodies showed no development, and after a few days these areas were covered by bacterial growth.

4. THE REVERSION OF THE L_1 INTO BACILLARY FORMS

While the successive steps of the transformation leading from the regular bacilli to L colonies were recognized, the author did not succeed in observing the mechanism of the reverse process, the appearance of *Streptobacillus* in the L_1 colonies. To study this process, blocks of agar cultures containing L_1 colonies were placed in different kinds of liquid media and after varying intervals were stained and studied under the microscope. Freshly isolated L_1 cultures usually revert to the *Streptobacillus* under such conditions, and the growth of the *Streptobacillus* appears as a small opaque papilla on the L colonies and later extends as a fluffy growth into the medium. The growth of *Streptobacillus* invariably started in only a few colonies. The largest number of bacillary colonies on an agar block of about 10 × 15 mm. was twelve. The youngest phase in the development of *Streptobacillus* observed microscopically was a small nest of bacilli spreading between the large bodies of the L colony. On the blocks as many as a hundred or more L colonies were present and each colony contained thousands of large bodies and granules of different sizes. The growth of the bacillus under the conditions prevailing in these experiments thus started in so few places that the chances to observe its derivation from the elements of the L colony were very small. In an effort to increase the rate of reappearance of the *Streptobacillus*, L cultures of various ages were inoculated into liquid media of varying composition and pH. These procedures exerted no influence on the appearance of the bacillus.

These negative results are not without significance. The L organisms revert to *Streptobacillus* in the same way no matter whether they are transplanted by picking isolated colonies or by transferring a large mass of culture. This indicates that the potentiality to revert to *Streptobacillus* is evenly distributed in the L colonies although the actual reversion may or may not occur when they are placed in liquid media. Such behavior corresponds much more to the development of a variant form than to cultivation of bacteria included in the colonies of an alien strain.

The reversion of the "old L_1" strain of Klieneberger was attempted under various conditions without success. A strain isolated by the author behaved in a similar way. The properties of this strain changed markedly when it became stabilized in the L_1 form. At first it was noticed that a few colonies in the cultures grew to a considerably larger size than the others and later the whole culture consisted of these large colonies. The strain both in macroscopical

and microscopical appearance became indistinguishable from the old strain of Klieneberger and did not revert again into *Streptobacillus*. The stabilization of this strain was not caused by purification but by a marked change in properties.

As variant forms in other bacterial species may lose their ability to revert to the original form, such observations furnish no evidence against the hypothesis that the L_1 is a variant of the bacillus. On the other hand, to bring into agreement the reversal of fresh strains with the symbiosis hypothesis, Klieneberger is compelled to assume that the fresh strains are not pure and carry the *Streptobacillus* enclosed in every colony. Such an assumption meets with many difficulties, as was pointed out in a previous paper (Dienes, 1939a). The bacillus is morphologically very different from the elements of the L_1 colonies, and its presence in the young L_1 colonies could not be missed in stained preparations.

In a recent paper Heilman (1941) described observations similar to those of the author concerning the reversion of L_1 into bacillary forms. These strains, after twelve months' cultivation and passing through 35 and 43 single colony fishing, still reverted to bacillary forms but they required successive transfer into two broth cultures and more time for this process than originally. Three strains lost the ability to revert to bacillary forms ten to twelve weeks after isolation.

5. GERMINATION OF THE LARGE BODIES IN CULTURES OF THE L_1

This process was observed both in cultures of freshly isolated strains and cultures of a strain marked "L_1 old," kindly supplied by Klieneberger. It was important for the success of this experiment to use cultures in appropriate phase of development and grown at room temperature. To avoid the tearing of the large bodies, transfers were made from the L_1 colonies by touching the surface of the medium with an agar block overgrown with L_1 colonies. After eight to twelve hours' incubation at 25 to 30°C. new growth started from many large bodies and structures similar to those visible in the photograph (plate II, 7–10) were produced, suggesting that the L_1 starts growing from the large bodies. Occasionally the breaking up of the large bodies into many small forms was clearly visible. These granules were similar in form and stained in the same way as the granules of the young L_1 colonies, and in some preparations the transition from large bodies to definitely growing colonies was apparent.

In the cultures of L_1 an accidental association between the large body and the young L_1 colonies is not excluded quite as well in the case of the *Streptobacillus* because the L_1 contains, besides the large bodies, other viable elements producing L_1 colonies. The similarity of the germinating large bodies in the cultures of L_1, of *Streptobacillus* and of some other bacteria makes it very probable, however, that the new growth starts from the large bodies in all these strains. In the photographs representing the germination of the large bodies of L_1 the shape and size of elements building up the young L_1 colonies is more clearly visible than in the photographs published in the previous paper (1939a).

Heilman (1941) describes the production of granules in the large bodies of L_1 and the observation of their growth with dark field in slide cultures. Of many

granules only a few were seen to reproduce, and the origin of the granules cannot be established with the method used; therefore Heilman's observations are only suggestive and cannot be regarded as a proof of the viability of the granules produced inside the large bodies.

6. AUTOLYSIS AND VARIATION OF STREPTOBACILLUS MONILIFORMIS

The culture of Strain 2 was observed to undergo a change which should be recorded because it helps to recognize the similarity of the processes connected with the development of L type of growth in different species of bacteria. *Streptobacillus* cultures in which many large bodies are produced have a tendency to autolyze. The majority of bacteria are not viable after forty-eight hours of incubation though transplants are successful for four to seven days. Cultures of Strain 2 were preserved in a CO_2 icebox at a temperature of -60 to $-70°C$. The properties of the cultures were unchanged up to about six months. On the plates inoculated with a culture preserved for twelve months a marked change was noticeable. Many bacterium colonies started to develop on the plate but they remained very small and were completely autolyzed after incubation overnight. Remnants of bacilli were visible in the colonies but they were not stained and did not grow in transplants. After further incubation, under the autolyzed bacterium colonies many L_1 colonies developed, and when a block of agar with these colonies was placed in ascitic broth the *Streptobacillus* reappeared in the culture. The bacteria transplanted from this broth to ascitic agar plates started again to multiply and produced tiny colonies, but they were completely autolyzed overnight. It was not possible to maintain this strain on ascitic agar plates by daily transplants.

Two more preserved cultures were available and were transplanted. In both cases many L_1 colonies developed on the plates together with a few large *Streptobacillus* colonies. The bacilli in these colonies were of regular shape and showed no tendency to autolyze or to form large bodies. Virulence for mice was very low. The strains cultivated from infected mice had the same properties as the injected bacteria. Whether the L_1 colonies grew from small autolyzed bacterium colonies or directly from the transplant could not be determined.

Early autolysis was observed in cultures of a *Flavobacterium* and of a few colon bacillus strains followed by the development of L type of colonies and the appearance of a stable variant. These cultures will be described in a later section of this paper. Their similarity to the autolyzing cultures of *Streptobacillus moniliformis* was pronounced, while the similarity to the usual cultures of *Streptobacillus* was much less apparent. A further point of interest is that a few autolyzing colonies often develop on plates inoculated from infected animals. As these colonies later are transformed into L_1 colonies they can be mistaken for L_1 colonies growing directly from the pathological processes.

7. L TYPE OF GROWTH IN THE CULTURES OF A FLAVOBACTERIUM

It was previously described (Dienes, 1939b) how tiny colonies similar in many respects to those of the L_1 of Klieneberger develop occasionally in cultures of

various bacterial species. These cultures presented exceptional properties in other respects also. In all of them many bacteria became swollen and were transformed into deeply stained large round bodies before the L type colonies appeared. These phenomena were most thoroughly studied in the cultures of a *Flavobacterium*, of *Escherichia coli* and of *Bacteroides funduliformis*.

In *B. funduliformis* (Dienes, 1941) the L strain could be isolated from the parent organism and maintained in a pure culture. It is strictly anaerobic, and both the young and fully developed colonies are in appearance and morphology similar to the L_1. The similarity of this strain to L_1 is obvious, and the problem is whether a similar relationship to the parent bacterial organism can be proven. Since this is now being investigated, this strain is not included in the present report. The recognition of the early stages of the L type of colonies in bacterial cultures has been made possible chiefly by the experience gained in studying a *Flavobacterium;* therefore, this strain will be discussed first.

Klieneberger suspected the presence of a "symbiotic organism" besides the *Streptobacillus moniliformis* in various cultures and recently (1940) she succeeded in isolating an L type of culture from a gram-negative bacillus isolated from guinea pigs. She relies for the recognition of the L type of growth on the large bodies and especially on the foam-like structure produced by autolysis of the large bodies. The methods which she uses, such as the examination of unstained cultures and the agar fixation method, do not allow the recognition of the young L colonies. These have a very characteristic appearance and can be easily recognized among the bacterial colonies in preparations made with the technique previously described. A further advantage of these preparations is that they contain the whole growth developing on the media, and large areas of the culture can be examined with the low and high power of the microscope. The characteristic appearance of young L type colonies is due to various factors. One is the pleomorphism of the culture. The colonies are built up of small granules and curved filaments showing all grades of transition to fairly large round or ovoid bodies. The pleomorphism is accentuated by the varying intensity of the staining and by the great fragility of the elements which are distorted by the slightest tearing of the colony. Another important characteristic of the colonies is that they extend not only on the surface but also grow down into the medium. The young growth of all the pleuropneumonia-like strains which the author isolated from mice, rats and human patients possessed these properties and could be differentiated easily from bacterial colonies. The bacteria, compared with the pleuropneumonia-like organisms, are relatively rigid. They are not deformed by tearing of the colonies. They are usually much larger in size and less variable in form and even if they grow beneath the surface of the medium the bacterial form is clearly visible. The appearance of young L type of colonies is very different from the fully developed and partly autolyzed colonies which are represented in the pictures of Klieneberger's papers (1938, 1940). For this reason the pictures illustrating the L type colonies in this paper cannot be compared with Klieneberger's pictures. The author published previously pictures of young L_1 colonies (1939a), and attention is called especially to photograph 9

of plate I and to photographs 4 to 8 of plate II of this paper. It is of great importance for the recognition of the L type growth in bacterial cultures to direct attention to the young L colonies because in many cases their development stops in this stage and the honey-comb-like structure which Klieneberger regards as characteristic of the L colonies does not develop.

The organism which is the subject of the present report is a small gram-negative rod which grows rapidly on plain or blood agar plates and produces large colonies. Its origin and bacteriological properties were previously described (1939b). It is pathogenic for rabbits, guinea pigs and especially for mice. Intraperitoneal injection of a few hundred organisms kills the mice in less than two days. They die also after application of the bacteria to the scarified skin. In guinea pigs, intracutaneous injection produces a chronic lesion without the development of generalized infection. The appearance of the culture, the production of a yellow pigment, and the fermentation reactions make it probable that the bacterium belongs to the genus *Flavobacterium*. A closer identification was not attempted.

At the time of isolation and in the first few transplants the cultures on solid media presented a characteristic appearance. In the thickly inoculated areas, large confluent colonies developed. These were surrounded with small colonies resembling the satellite growth of influenza bacilli around a staphylococcus colony. In some plates large areas were covered with very tiny colonies which in the neighborhood of large colonies developed later into satellite colonies. Repeated transplants from the large and small colonies always produced both colony types and it became evident during the study of this strain that the two types of colonies are variants of the same strain. After repeated transplants large colonies developed on the whole inoculated area, and all attempts to regain the original growth form were unsuccessful. Early transplants of the strain are kept frozen in a CO_2 icebox and thus far reproduce both the large and the small colonies. Passage through mice and guinea pigs does not influence the characteristics of the culture. If animals are injected with a fresh culture producing both types of colonies, the strains regained from them behave in a similar way. In successive animal passages the property of producing small colonies was lost in the same way as on artificial media. This property is not connected with the virulence of the strain in any way.

In stained preparations marked differences were present between the large and small colonies. The large colonies consisted of small bacilli of uniform shape and staining. The small colonies consisted of pleomorphic diphtheroid-like forms which by swelling were transformed to large oval bodies. After twenty-four hours these colonies autolyzed and after forty-eight hours usually did not grow in transplants. In many colonies before autolysis a secondary growth, similar in appearance to the L_1 growth, started to invade the agar.

The illustrations in plate I will give an impression of the actual observations. Photograph 1 shows a blood agar plate inoculated from the peritoneum of an infected mouse. The thickly inoculated area was covered with large confluent colonies. Outside this area many tiny colonies were growing. Photograph 2

shows a stained wet agar preparation of the large and small colonies after three days' incubation. The large colony is stained around the periphery only. The small colonies are deeply stained and consist mainly of the L type of growth. Photograph 3 shows the edge of a large colony consisting of regular small bacilli. Photograph 5 shows a small bacterial and an L type of colony. The bacterial colony is situated on the surface of the medium and consists of large pleomorphic bacilli. In the photographs it is not apparent that the L type of colony invades the agar and the elements of the colony are too small to be clearly visible. However, the whole appearance of this colony is very different from both the small and large bacterial colonies. Photograph 6 represents a further stage in the development of the small bacterial colonies. At the periphery of the colony several large swollen forms are visible. Photograph 8 shows a small autolyzed bacterial colony in which only a few swollen bacterial forms remained, and at three different sites the secondary L type of growth had started. This colony is reproduced with very high magnification (1:3000) to show the form of the morphological elements of the growing L colonies. In photographs 9, 10 and 11 small L type colonies are reproduced with the same high magnification (1:3000). This growth is pleomorphic and consists partly of very small elements but it does not differ in any essential character from bacterial growth. The photograph does not give a truly adequate impression of the appearance of the L type growth, partly because in the dry preparations the form is not maintained as well as in wet preparations and partly because in the absence of the third dimension it is impossible to see the actual arrangement of the elements in the colonies. Photograph 10, for instance, represents a coiled fine filament penetrating the medium, but it is difficult from the photograph to get an adequate impression of the actual structure.

The autolysis of the tiny bacillary colonies made it possible to obtain the L type of growth in pure culture. After forty-eight hours of incubation transplants made from the tiny colonies no longer gave the bacterial type of growth. If an agar block with small colonies was transferred into the broth, the medium remained sterile indefinitely. However, on the agar block itself, the L type of colonies increased considerably in size. When agar blocks with the tiny colonies were cut out and placed on new media so that the tiny colonies touched the new medium, the L type colonies increased in size in the original block and an abundant growth of these colonies developed in the medium beneath the agar block. Photograph 7 shows the growth obtained in transplant and stained with methylene blue. The magnification of this photograph is the same as that of photograph 2, which represents the colonies used for the transplants. It is seen that the L colonies in the transplant reach a considerable size compared with the size of the original colonies. A second transplant made in the same way gave just as abundant growth. In the third transplant only a few colonies developed and growth was not obtained in further transplants. The first transplant grew equally well on plain, ascitic fluid and horse-blood agar plates. To make transplants from the L colonies, different methods were tried. When the surface layer of the media together with the colonies was scraped off and spread

on new medium no growth was obtained. With the L_1 this method gives a good growth even from a few colonies. If the inoculated surface was covered with an uninoculated agar block a few colonies developed under the larger pieces of agar transferred from the original culture but never in other places. Cultivation in the presence of CO_2 or under anaerobic conditions exerted no influence. The colonies grown in transplants are very similar to the young colonies of the L_1 just before the surface layer of the large bodies develops.

Although the L type colonies were isolated from the culture of the *Flavobacterium* in pure culture, the conditions which secure continuous growth were not recognized. One of the main difficulties in the study of L type colonies of the *Flavobacterium* was that in only a few cultures was the growth of the small colonies sufficiently abundant to initiate growth in transplants.

The observation previously mentioned that transplants made from the small and large bacterium colonies give a similar growth indicates that the atypical colonies do not represent a different strain but a different growth form of the same strain. Further evidence was obtained on this point by the investigation of the development of the cultures. When a large colony consisting of regular small bacilli was transplanted, the majority of the bacilli, after some incubation, became swollen to form large round bodies, and the new growth started in the form of large pleomorphic bacilli. The initial phase of growth was the same both in the thickly and thinly inoculated areas. Photograph 4 represents an area of the medium inoculated with regular small bacilli after incubation overnight at room temperature. In thickly inoculated areas which later were covered with large colonies, small regular bacilli appeared after some growth, and in a short time overgrew the pleomorphic forms. In the thinly inoculated area this did not occur; the pleomorphic bacilli, however, soon stopped growing and were later autolyzed.

For a short period after isolation the culture had a tendency to grow in the form of large pleomorphic bacilli, to autolyze and to produce L type colonies. This tendency was present also in the small regular bacilli which, transplanted to new media, were transformed into the pleomorphic form. After some cultivation this tendency was completely lost and the small regular bacillary forms remained stable. All attempts to restore the tendency toward pleomorphism and production of L type of colonies were unsuccessful. The inefficiency of animal passage in this respect has already been mentioned. Cultivation in liquid and solid media of various compositions and pH exerted no influence. When the strain was isolated it was apparently in a condition of increased variability and this was lost in cultivation. In cultures kept frozen in the CO_2 icebox, as was already mentioned, the variability has been maintained for over a period of one and a half years.

The phenomena observed in the culture of the *Flavobacterium* are very similar to those previously described in cultures of *Streptobacillus moniliformis*. When the small L type colonies begin to develop they are very similar and can hardly be differentiated by their appearance in the cultures of these two strains. In both cases the development of L type colonies is preceded by the swelling of the

bacteria into large round bodies and the autolysis of the bacterial colonies. It has already been noted that *Streptobacillus* may grow in the form of small autolyzing colonies which are similar in every respect to the small colonies of *Flavobacterium*. In *Flavobacterium* the surrounding of the bacillus exerts a large influence on its development. In thickly inoculated areas small regular bacilli develop without pleomorphism and without L type colonies. The tendency to produce these phenomena, as has already been remarked, is present in the bacilli but cannot manifest itself while they are in the large colonies. In *Streptobacillus* large bodies develop not only in the small but also in full sized colonies. The crowding inhibits a further part of the transformation process, that is the germination of the large bodies. The transformation process in both species is essentially similar but it is modified in a different way by secondary influences.

8. L type of growth in colon bacillus cultures

The L type of growth was discovered in the cultures of colon bacillus by the study of a large number of routine bacteriological cultures. This growth was always associated with the appearance of large swollen bacterial forms in the cultures, and the presence of these helped to select the cultures appropriate for study. A few large bodies are often present in cultures isolated directly from pathological urine specimens. These cultures show no other peculiarities, and the large bodies disappear in transplants. In a few cultures the majority of bacteria are transformed into large round bodies. These are very fragile, and in the usual dry smears they are disfigured and often cannot be recognized. Strains of this type are very rare. In a period of two-and-a-half years about twelve such strains were found among 4,000 to 5,000 colon bacillus strains isolated from urine. Sometimes within a few weeks several such strains were found, sometimes none for many months. In strains isolated from stools large bodies were never seen. L type growth developed only in cultures which produced many large bodies, but not in all such cultures.

The cultures in which many large bodies develop can usually be recognized by their appearance. The colonies vary in size and appearance, and the small colonies have a tendency to autolyze. The development of large mucoid colonies besides tiny autolyzed colonies is usually a sign of the large bodies. The cultures do not belong to a special variant form; they are rather characterized by excessive variability. On the same plate mucoid, smooth and rough colonies may be present, and the large bodies develop sometimes from regular-shaped bacilli and sometimes from long wavy filaments. This excessive variability lasts for a certain period, but sooner or later is lost in cultivation. In broth cultures it disappears usually in the first passage. The strains at the time of isolation, as described in the case of *Flavobacterium*, are in an exceptional condition. The only way of maintaining this condition was to keep the cultures frozen in a CO_2 icebox.

The fermentation reactions of some of these strains immediately after isolation were not typical. Some of the cultures were colorless on Endo medium and fermented lactose only after several days. After a few transplants all strains fermented lactose promptly and produced red colonies on Endo medium. A

more accurate classification of the strains was not made. The detailed description of two strains will show that the cultures were pure and that the different types of colonies visible on the plates were produced by a single strain.

Large bodies were described by several authors in cultures of colon bacilli and related bacteria (Bergstrand, 1923; Mellon, 1925; Klieneberger, 1930). Nyberg (1938) studied an aerogenes strain cultivated from urine, the variability of which was in many respects similar to the strains described in this paper. The appearance of these cultures is similar in many respects to the growth developing after lysis with bacteriophage. Whether these strains contained a phage and whether this played a rôle in the observed phenomena has thus far not been studied.

The present report is based mainly on the study of two strains which retained their variability for a longer period than the others. Strain 1706 was cultivated from the urine of a patient on two consecutive days. The urine specimen is preserved in the CO_2 icebox and thus far has continued to give cultures similar to the original. On blood agar and Endo plates inoculated with the urine the growth starts in the form of small mucoid colonies. In densely inoculated areas the colonies remain small, and after incubation overnight they are autolyzed and are resorbed into the agar. Near the periphery of the inoculated area the colonies develop to a larger size, and they are transparent and of a watery consistency. The culture is similar in appearance to a type III pneumococcus culture. During the second day small mucoid papillae appear in many autolyzed colonies. The secondary growth is again autolyzed and resorbed, with the exception of a few colonies which develop into large, opaque, mucoid colon bacillus colonies.

After a few hours of growth the young colonies consist of small regular gram-negative bacilli widely separated from each other by an abundant accumulation of mucoid material. At the height of development the bacteria gradually enlarge and are transformed into spherical bodies of 3 to 10 microns in diameter. At the same time the colony dries and flattens out and apparently is resorbed into the agar. This process is caused by the autolysis and resorption of the mucoid material which is present in abundance in the young colonies; the bacteria transformed into the large bodies remain intact for a while. Later the large bodies develop vacuoles, or the protoplasm breaks up into stainable or non-stainable granules. At first this process produces a foamlike structure similar to the structure of an autolyzed *Streptobacillus moniliformis* or L_1 colony. Finally, a granular mass, stained pink by methylene blue, remains on the site of the colony. The mucoid secondary growth consists at first of regularly shaped bacteria or bacterial filaments which pass through the same transformation as the original culture. The stable large colonies consist of regular small bacilli which are not transformed into large bodies either in the original culture or in subcultures. Transplants from a colony in which the transformation into large bodies is completed grow very poorly. The growth, as far as could be determined, starts from the few regular-shaped bacilli which remain in the culture.

The purity of the strain was established by repeated picking of isolated

colonies. These always reproduced the whole scale of variation, including the large stable colonies. If the strain is transplanted by picking small mucoid colonies, its characteristics are retained for several weeks with little change. If the large stable colonies are included in the transplant, they overgrow the whole culture. From the broth cultures usually only the stable variant form is cultivated. Though the variability of this strain persists longer in cultivation than the variability of most other strains, it slowly diminishes. The colonies grow larger; the formation of large bodies and the autolysis come later and are less complete. Slowly the strain approaches the properties of the stable variant.

When the large bodies are fully developed, the L type of growth starts in the agar beneath the colonies. At first only a few tiny isolated colonies are noticeable below the bacterium culture. Later, the agar beneath the bacterium colony is filled out densely with the L type of growth. At first this growth consists of deeply stained fine filaments and granules. Later, some of these elements swell up into fairly large forms, while others degenerate and disappear. At this stage the agar beneath the autolyzed colony seems to be filled with stained granules and peculiar forms, the identity of which can be established only by following their whole development.

It is probable that an adequate impression of the macroscopical appearance of the cultures can be formed from their description. Therefore, in order to keep down the number of photographs only the microscopical structure of the colonies is illustrated. Photograph 1 shows a young colony consisting of regular bacilli pushed apart by an abundance of mucoid material. The gradual tranformation of small bacilli into the large bodies and the fully developed large bodies are illustrated in photographs 2 and 3. Many of the large bodies in photograph 3 contain vacuoles. Their similarity to the large bodies of *Streptobacillus* and L_1 is apparent. It has already been mentioned that the large bodies of the colon bacillus after Giemsa staining show the same type of nucleus-like chromatin granules described by Ledingham in the large bodies of the organism of bovine pleuropneumonia. In photograph 4 the large bodies have partly lost their regular shape and at the edge of the colony a new growth appears consisting of small pleomorphic forms. Photographs 5 to 12 show the appearance of this growth. In photograph 5 the bacterial colony was washed off the agar and the small colonies developing under the bacterial growth are visible. Photograph 6 shows a small bacterial colony which was autolyzed and only a few large bodies and the L type of growth are visible at the site of the colony. The large regular bacterium which is visible in the photograph was carried there during the staining process from a neighboring colony. The small granules, the filaments and the swelling of these to fairly large pleomorphic forms is visible in the rest of the photographs. One of these, photograph 10, is highly enlarged (1:3000). The photographs, as in the case of *Flavobacterium*, do not give as clear impressions of these structures as the examination of wet stained preparations. The uniform bright blue color of all the bacterial structures, such as the regular bacilli, of the large bodies and of the L type of growth helps considerably to distinguish the L type of colonies from unorganized precipitates which take a dull violet staining.

The most important evidence which indicates the organized nature of the L type colonies is the observation that they are produced like the L_1 colonies of *Streptobacillus* by the germination of the large bodies.

The direct connection between the large bodies and the L type of growth was observed with this strain first on a blood agar plate inoculated from a broth culture. Two boiled-blood, ascitic-fluid, broth tubes were inoculated with agar blocks containing small autolyzed colonies. For two days the cultures showed no turbidity. At this time transplants were made on blood agar plates and these were examined at short intervals. The transplant from one tube showed an abundant growth of regular small bacilli. In the transplant from the other tube only a few bacterial colonies started to grow, but many large round bodies were visible on the surface of the agar. After eight hours' incubation small granules and fine filaments grew into the agar from the large bodies and after twelve and twenty-four hours tiny L type colonies were visible around them. These L colonies developed exclusively in association with the large bodies. They never developed alone or together with regular bacilli. The only elements which produced the L type of colonies were the large bodies in the broth culture. The L type colonies reached their maximal development in a short time, and their size did not increase after twenty-four hours. Many broth cultures were examined but none, except the one just described, contained appreciable numbers of large bodies.

Later it was found that the germination of the large bodies can be easily observed if colonies grown on blood agar plates are transplanted when the autolysis just begins and the first L type of colonies begin to develop beneath the bacterium colonies. For cultures grown at room temperature (20 to 25°C.) the appropriate time for transplanting is between sixteen to twenty hours. Growing cultures at room temperature, as in the case of *Streptobacillus*, gives more consistent results than growing at 36°C. Photographs 1 and 2 on plate IV show two tiny colonies associated with the large bodies after twenty-four hours' incubation. These pictures are not sufficiently representative because they do not show distinctly the form of the small elements which build up the L type of growth. The pictures illustrating the germination of the other colon bacillus strain, to the discussion of which we turn now, show the elements of the L type of colonies better.

The other colon bacillus strain (12395) in which the whole cycle of transformation was observed presented properties markedly different from strain 1706. It grew abundantly in large colonies both on blood agar and Endo plates. The only peculiarity of the cultures was an excessive stringiness. Growth could be separated from the medium only with great difficulty. Endo medium turned red. After several transplants the colonies were more mucoid and less tenacious and occasionally their autolysis was observed. The young colonies consisted of regular large bacilli separated by abundant mucoid material. After twenty-four hours of incubation the bacteria in the center of the colonies were swollen, and in the next twenty-four hours a large proportion of the bacteria were transformed into large spherical or fusiform bodies. The L type of growth did not

develop beneath the colonies, but in transplants, after a few hours' incubation, tiny L type colonies were often seen among the young bacterial colonies. Transplants made with a platinum loop were quickly overgrown with bacteria, and the L type of colonies did not develop with sufficient frequency to observe their origin. A small change in the technique brought success. Squares with the viscid colonies were cut out from a blood agar plate and impressions of the colonies were made on blood agar plates. No visible culture material remained in the inoculated plates, but in stained preparations many large bodies were visible on the agar surface. The large bodies after an hour's incubation at 36°C. showed no change. After five and a half hours the L type of growth started to develop from about every second one of the bodies. Such growth was not visible except in connection with the large bodies. At the same time a few bacterium colonies started to develop. In a preparation made after incubation overnight at room temperature, out of seventeen large bodies counted, twelve produced L type of growth. The success of this experiment depended mainly upon the condition of the culture from which the transplants were made and upon the absence of abundant bacterial growth in the transplants. It was found advisable to transplant cultures grown at room temperature after varying periods of incubation.

Photograph 3 on plate IV illustrates the large bodies which are connected by transitional forms with the normally shaped bacilli. In photographs 5 to 7, in which the L type of growth has just started, the morphological appearance of this growth is clearly visible. Photograph 4 represents a further development of the L type of colony. The L type of colonies rarely reached a larger size in this strain. All photographs were taken from dry preparations and the germinating large bodies are slightly distorted.

Cultures 1706 and 12395 are different in appearance but on closer examination present many similarities. In both, large amounts of mucoid material are produced; the bacteria gradually swell up into large round bodies which, under appropriate conditions, develop into L type colonies. The differences between the strains are quantitative rather than qualitative. In strain 12395 the mucus is very tenacious and does not autolyze regularly, the bacterial growth is not inhibited in the colonies, and the large bodies do not germinate while they are in the colonies. In the colonies of strain 1706 the large bodies develop early, the growth of the bacteria is inhibited, and the L type of growth is not inhibited. At the edge of the seeded area, probably in consequence of the better diffusion of metabolic products, the colonies of the 1706 strain develop to a larger size and autolyze later. These colonies in microscopical appearance are very similar to the colonies of strain 12395. After many transplants the culture of strain 1706 is more similar to the cultures of strain 12395, both the autolysis and the production of the L forms being decreased. The potential development of the two strains is similar, except that the tendency toward autolysis and variation is more pronounced in one than in the other.

This can also be said of the other strains in which large bodies were produced in abundance. The arrest of the growth followed by tranformation into large

bodies, autolysis and development of L type of growth were the fundamental observations in all cases. These phenomena developed in variable degree not only in the cultures of the different strains but also in different colonies of the same strain. Experimentation with most strains was difficult because after a few transplants the stable variant form replaced the variable forms even if the transplants were made from the small variable colonies. It is essential to study several strains because only a few show the phenomena described in a form accessible for study.

The cultivation of the L type of growth in successive transfers has been unsuccessful thus far. The autolysis of the cultures was not complete and the transplants were overgrown by bacteria in every case.

With the colon bacillus strains the influence of the media on which they grow is more marked than in the case of *Streptobacillus moniliformis*. L type colonies were observed in the cultures of several strains on blood agar plates only and not on Endo medium or plain nutrient agar. The influence of the medium was most thoroughly studied with strain 1706. This strain immediately after isolation went through the same transformation and produced the L type of growth abundantly on blood agar, ascitic agar plates and Endo medium. After a few transplants the tendency of the strain to autolyze and form the L type of colonies was somewhat diminished. The influence of the medium was studied at this time. The strain has grown well on simple water agar without addition of nutrient substances. If peptone, ascitic fluid, egg white, glucose or sucrose were added to the water agar, the growth was abundant and large mucoid colonies developed. These autolyzed after a few days but neither large bodies nor the L type of growth was produced by them. On nutrient agar L type colonies developed only on slightly acid hard plates (pH 6.5). If the plates were made soft by the addition of an equal amount of broth, or if the pH were changed to 7.2 or 8, the bacterial growth remained excellent, but no L type colonies developed. It is interesting that in the case of *Streptobacillus* alkalinity was most favorable to the development of the L type of colonies, while in the case of the colon bacillus slight acidity was preferable. Cultivation under anaerobic conditions or in partial CO_2 exerted no noticeable influence. Apparently in the case of colon bacilli certain substances present in broth, horse blood and ascitic fluid considerably help the development of the L type colonies and by favoring the transformation of bacteria into large bodies may inhibit bacterial growth.

A few experiments have been made to study the influence of lithium and chromium salts. These salts have been used with success to produce bacterial variants, especially large, swollen forms (Klieneberger, 1930; Sander, 1938). These salts exerted no favorable effect on the germination of the large bodies of *Streptobacillus moniliformis*. Addition of 0.01% potassium bichromate seemed to favor in certain plates the germination of the large bodies produced by colon bacillus strain 12395. However, it is probable that the inhibition of the bacterial growth was the main effect and the increased germination of the large bodies was probably only apparent. The L type of growth remained very slight. Addition of .5% lithium chloride to broth cultures in a few experiments did not

favor the production of large bodies. It is desirable to study more systematically the effect of certain media which apparently favor the development of the large bodies (Reed and Carr, 1923; Wahlen and Almaden, 1939) and also the effect of salts. It would be of great help for the study of the L growth and the connected phenomena if their production could be artificially induced.

The comment made on the fundamental similarity of the phenomena observed in the cultures of *Flavobacterium* and *Streptobacillus moniliformis* applies also to the colon bacillus cultures.

DISCUSSION

According to the observations described in this paper, the large bodies developing in bacterial cultures fulfill a special function. They not only germinate under appropriate conditions but their descendants produce a growth very different from the parent organism. The development and properties of this growth were most thoroughly studied in cultures of *Streptobacillus moniliformis*, and can be briefly described as follows: The regular bacillary forms are transformed into large round bodies by gradual swelling. These, in the colonies, do not multiply and soon autolyze. If they are transplanted under appropriate conditions they germinate by growth of small granules and diphtheroid-like forms penetrating into the medium. These elements are very small, but examination of photographs 5 to 7 in plate II shows that their morphology does not differ essentially from that of bacteria. The multiplication of these small forms produces the L_1 colony. In a more advanced stage the small forms are transformed by swelling into large round bodies which again may germinate or break up into numerous small granules. The morphology of the bovine pleuropneumonia group of organisms is very similar to that of the L_1. According to observations of Klieneberger, and Dawson and Hobby (1939a, b) the L_1 remains serologically similar to *Streptobacillus*. Freshly isolated L_1 strains in liquid media usually revert to *Streptobacillus*.

This description of the development of L_1 contradicts in several points Klieneberger's opinions. According to her, the large bodies of L_1 and of the bacteria are not similar and the large bodies in the cultures of *Streptobacillus moniliformis* are produced by the symbiotic L_1 and not by swelling of the bacteria. These points have been already discussed. The actual observations of Klieneberger and the author concerning the morphology of L_1 are very similar. Both observed that the cultures consist at first of small granules and fine curved filament which gradually swell into large round bodies. Both the small and large elements differ from the bacteria by their softness. The author's impression is that the most important difference between the L_1 and the usual bacterial forms is the regular transformation of the granules in the L_1 into large bodies and multiplication by germination and breaking up of these bodies. This difference is not fundamental because many bacteria produce similar large bodies. The filtrability of the granules does not seem to be of importance because size is one of the most unreliable criteria for differentiation of microorganisms, and some bacteria are nearly as small as the granules of L_1. The author has never seen branching filaments in the cultures of *Streptobacillus moniliformis* or of the L_1.

Observations similar to those made in the cultures of *Streptobacillus moniliformis* have been made in a more less complete form in many other bacterial species. In a bacillus, which Klieneberger isolated from guinea pigs, and in *B. funduliformis* the L colonies have been isolated, and are morphologically very similar to the L_1. Their derivation from the bacillary forms, their serological properties and their eventual reversion to regular bacillary forms have not yet been studied. In *Flavobacterium* the transformation of the bacteria into large bodies and the development of L type of colony has been observed. The L type of colonies could be kept growing in pure culture only in three generations. In the cultures of *H. influenzae*, of colon bacilli and of gonococci large bodies and tiny L type of colonies have been seen. The germination of the large bodies has been observed in two strains of *E. coli* and in one strain of *H. influenzae*. The L type of growth has not yet been isolated in pure culture in these strains. Those parts of the transformation process which were observed were so similar in the different species that it cannot be doubted that they indicate the development of an analogous process.

In all species only a few exceptional strains produced L type of colonies, and they retained this property only for a short period. The strains seemed to be in a condition of exceptional variability. This is probably true also of *Streptobacillus moniliformis* in which species apparently all strains can be induced to produce L_1 colonies. Strains cultivated from the pharynx of healthy rats usually show no or little pleomorphism, and the pleomorphic strains sooner or later lose this property. Strains of *B. funduliformis* cultivated from severe infectious processes are usually pleomorphic and two out of three such strains produced L type colonies. Very similar strains cultivated from the uterine cervix showed no pleomorphism. All strains which produced L type colonies were isolated from infectious processes. For instance, L colonies were never produced by colon bacilli cultivated from stools or by influenza bacilli cultivated from the throat. The connection, however, between infection and L type colonies is obscure because most strains isolated from infectious processes do not produce L type of colonies, and in a single case, that of *Flavobacterium*, it has been observed that the loss of ability to produce L colonies was not accompanied by a change in virulence.

In the case of colon bacilli it was apparent that the production of the L type growth is a part of the variability of the bacterium. The strains which produced L type colonies presented an extreme variability in several instances. Such observations suggest that the production of large bodies and L type colonies may represent the final stage of bacterial variation. The rough form may be a transitional stage in this process. The fact that in all cultures in which many large bodies were produced the usual multiplication stopped and the cultures autolyzed is in agreement with this conception. When the large bodies germinated they produced a growth different from the parent organism. The development of the large bodies changed the potentiality of the bacteria for further development.

Transformation into large bodies can be induced by cultivation of bacteria on deficient or slightly toxic media. Addition of lithium, calcium or chromium

salts to the media is the most effective method. Under such conditions usually only a small proportion of the bacteria are transformed into large bodies and the tendency to produce them is lost as soon as the bacteria are brought back to normal media. The same influences which favor the production of the large bodies also increase the variability of bacteria.

The development of large bodies in the strain described in this paper did not seem to be induced by injurious influences, but was rather an inherent tendency of the strains. It occurred on media best suited to the strains, and it was to a large extent independent of the composition of the media. The transformation involved the majority of the bacteria in several consecutive transplants. The initial growth of these cultures was vigorous, and several of the species studied, such as the colon bacillus and *Flavobacterium* are not sensitive even to considerable changes in the composition of the media. The strains may have been exposed to injury in the host, but the properties of the large bodies and of the L type of growth do not seem to indicate a degeneration of the cultures. The L_1 form, when it is well established, grows nearly as abundantly as the parent organism; it is considerably less exacting as to the composition of the media and has a higher heat resistance. The main difference between the L_1 and the parent strain is probably, as has already been explained, the multiplication of L_1 by formation and germination of the large bodies. It is improbable that such a process represents a degeneration of the culture. It is more likely that certain toxic influences awaken a latent tendency which shows itself only rarely under normal conditions.

If the L type of colonies represent a symbiont in the cultures as Klieneberger believes, the observations described in this paper have no important bearing on the general biology of the bacteria. If they represent a variant form, the observations indicate that the variability of bacteria extends much further than is commonly supposed. The bacteria apparently are able to grow in the form of soft granules and to multiply by the development and germination of large bodies. Furthermore the bacteria pass through periods of increased variability and produce L variants only during such periods.

It has previously been pointed out that the desire to avoid such far-reaching conclusions probably induced Klieneberger to assume a symbiosis between the bacteria and the L_1. The author's impression is that the information already available decides against such assumptions. It is difficult to bring into agreement with the symbiosis hypothesis such facts as the constant association of L_1 to the *Streptobacillus moniliformis*, persisting even through animal passage, the seriological similarity of L_1 and of the bacillus and the reversion of the L_1 into bacillary form. The suppositions which Klieneberger makes to avoid these difficulties are not convincing and are not supported by direct experimental evidence. The nature of the L type of growth will probably be entirely cleared up by thorough study of similar forms in several bacterial species and it is not necessary to discuss it now in detail. The author wishes only to point out again that this question has great theoretical importance. If the bacteria can grow in variant forms similar to the L_1, we have to face entirely new problems in their study.

The nature and functions of the large bodies are probably not entirely cleared up by the observation of their germination. The observation has been repeatedly described that the large bodies under certain circumstances reproduce bacteria of the usual type. The description of this process is in no case convincing, but, as the author himself has observed it in a single instance (1939c), he has no doubt that it occurs occasionally.

SUMMARY

By examination of a large number of routine bacteriological cultures a few strains which produced large bodies in abundance were observed in many species. In all species, including *Streptobacillus moniliformis*, the morphological appearance of the large bodies was similar, and they were produced by gradual swelling of the bacteria.

In cultures of *Hemophilus influenzae*, *Escherichia coli* and *Streptobacillus moniliformis* the germination of the large bodies was observed. Their descendants are different from the parent organism and are similar both in morphology and development to the pleuropneumonia group of organisms. Such "L type" of colonies were observed in cultures of a *Flavobacterium*, of *Bacteroides funduliformis* and of the gonococcus in addition to the three species already mentioned. They were isolated in pure cultures from *B. funduliformis* and from the *Flavobacterium*.

Strains which produced L type of colonies are rare in most species. They are in a transient stage of increased variability. A strain of *Flavobacterium* and two strains of colon bacilli presenting these phenomena have been described in detail.

The significance of these observations depends mainly on whether the L type of colonies are genetically connected with the bacteria. The cultures of *Streptobacillus moniliformis* were further studied in connection with this question. The author's previously expressed conclusion that the L type of growth is a variant of the bacteria has remained unchanged.

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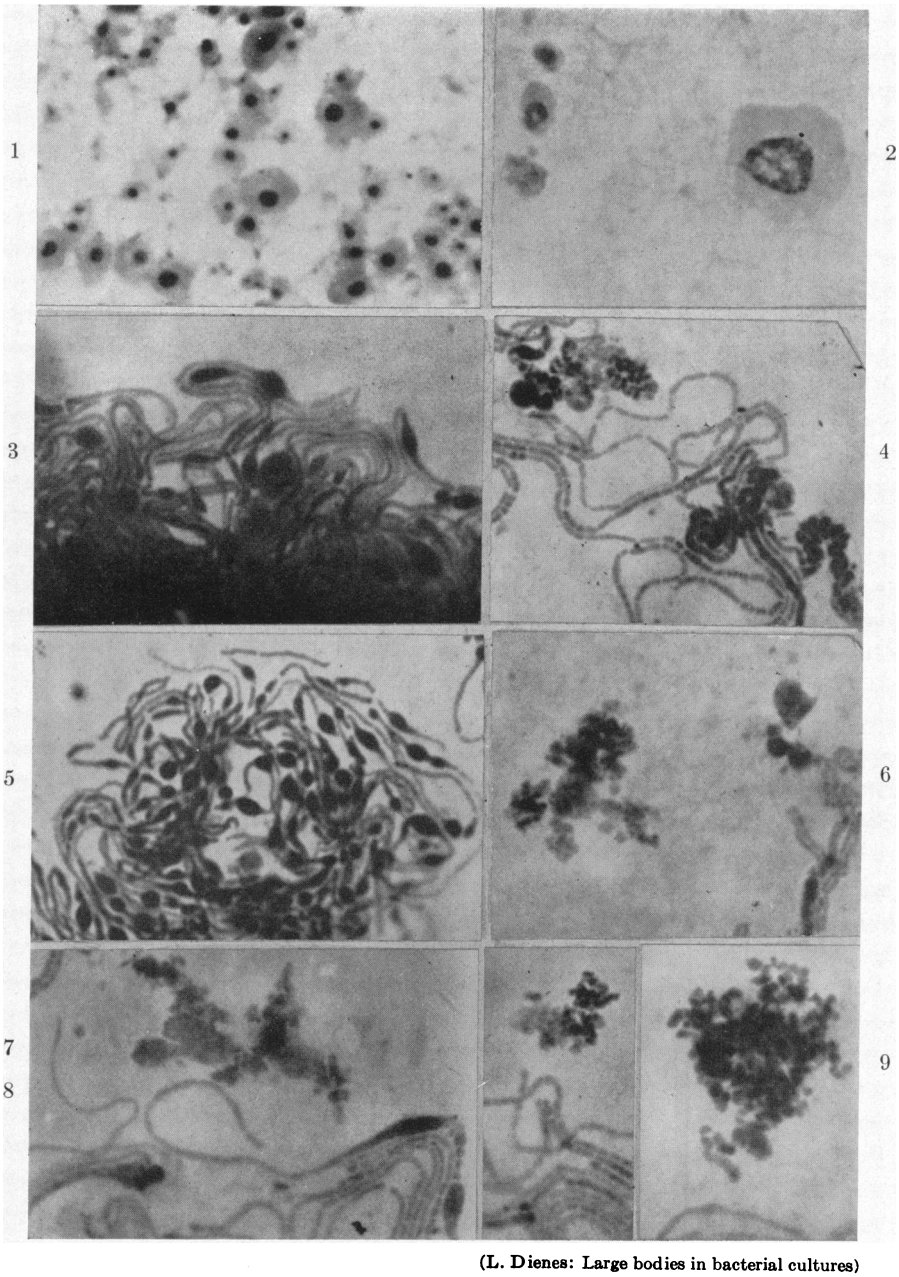
PLATES

PLATE I

Photographs 1 and 2 were made from dried impression preparations fixed with methyl alcohol and stained with Giemsa stain; 1, represents the "nucleated" large bodies of L_1; 2, a small and a very large such body from cultures of colon bacillus strain 1706. (× 2000.)

Photographs 3–9 are from dry agar films stained with methylene blue and azure with the technic described in the text. (× 1800.) Photograph 3 represents the edge of a streptobacillus colony of Strain 3 grown 24 hours at room temperature; 9, a small L_1 colony from the same plate; 4, 6, 7 and 8 represent the cultures after 12 hour incubation. Photograph 5 is a 24 hours old colony of Strain 2 on boiled blood agar without ascitic fluid.



(L. Dienes: Large bodies in bacterial cultures)

PLATE II

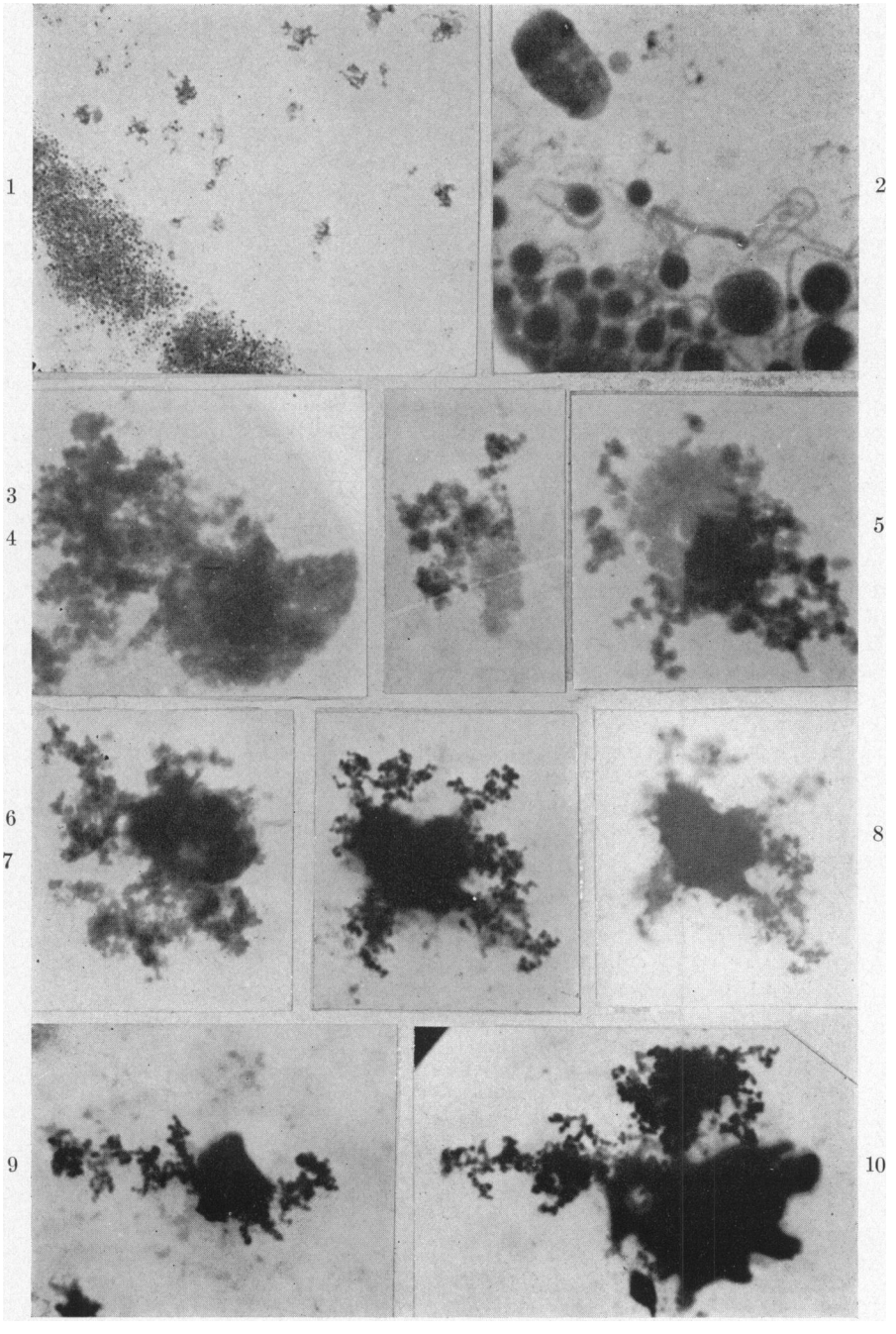
Germination of large bodies in cultures of Strain 2 of *Streptobacillus moniliformis* and of the L_1.

Photograph 1 was made from an ascitic agar plate inoculated with streptobacillus and incubated for 24 hours. The inoculum in the thick streak is unchanged; in the lightly inoculated areas tiny colonies have developed. Dry agar film stained with methylene blue and azure. (× 90.)

Photograph 2 represents a part of the thickly inoculated area with high magnification. (× 2000.) A large body on the left upper corner is considerably increased in size.

Photographs 3, 4, 5 and 6 show the small L type colonies from the same plate. The growth starts from large bodies which are very much increased in size (especially in 3). In 5 the growth starts from a small group of large bodies. Magnification of 3 and 5 is × 2000, the others × 1700.

Photographs 7, 8, 9 and 10 show the growth starting from the large bodies of Klieneberger's L_1 strain. Dry agar film stained with toluidin blue and thionin. (× 1700.) Photographs 7 and 8 show the same tiny colony. In photograph 7 the focus is set below the surface of the agar and the growth extending into the agar is visible. The large body is out of focus. In photograph 8 the focus is set on the large body, which is slightly disfigured. The growth below the agar surface is out of focus. The tiny colony represented in photograph 10 started to grow from a group of large bodies.



(L. Dienes: Large bodies in bacterial cultures)

PLATE III

Flavobacterium

Photograph 1 shows a blood agar plate inoculated with the peritoneal exudate of an infected mouse (plate I). The areas thickly inoculated are covered with confluent large colonies. The colonies on the areas thinly inoculated are very small. (Magnification about × 5.)

Photograph 2 was made from a stained wet agar preparation from the same plate. A large colony, visible in the photograph, is stained only at the periphery. The small colonies are deeply stained. (× 90.)

Photograph 3 shows the edge of a large colony. Dry agar film stained with methylene blue and azure. (× 2000.)

Photograph 4 represents a thickly seeded area of a blood agar plate incubated overnight at room temperature. The majority of bacteria swelled up into large round bodies. Multiplication started at a few places only. Dry agar film. (× 2000.)

Photograph 5 represents a tiny colony consisting of pleomorphic bacilli and an L type of colony situated under the surface of the medium. Dry agar film. (× 2000.)

Photograph 6 a tiny colony of pleomorphic bacilli. Dry agar film stained with toluidin blue. (× 1700.)

Photograph 7. L type of colonies grown in a transplant from plate I. The colonies have grown larger than in the original plate. Wet agar preparation stained with methylene blue and azur. (× 90.)

Photograph 8. An autolyzed tiny bacterium colony from plate I. A few large bodies are maintained and L type of growth started in two places below the surface. (Very high magnification × 3000.)

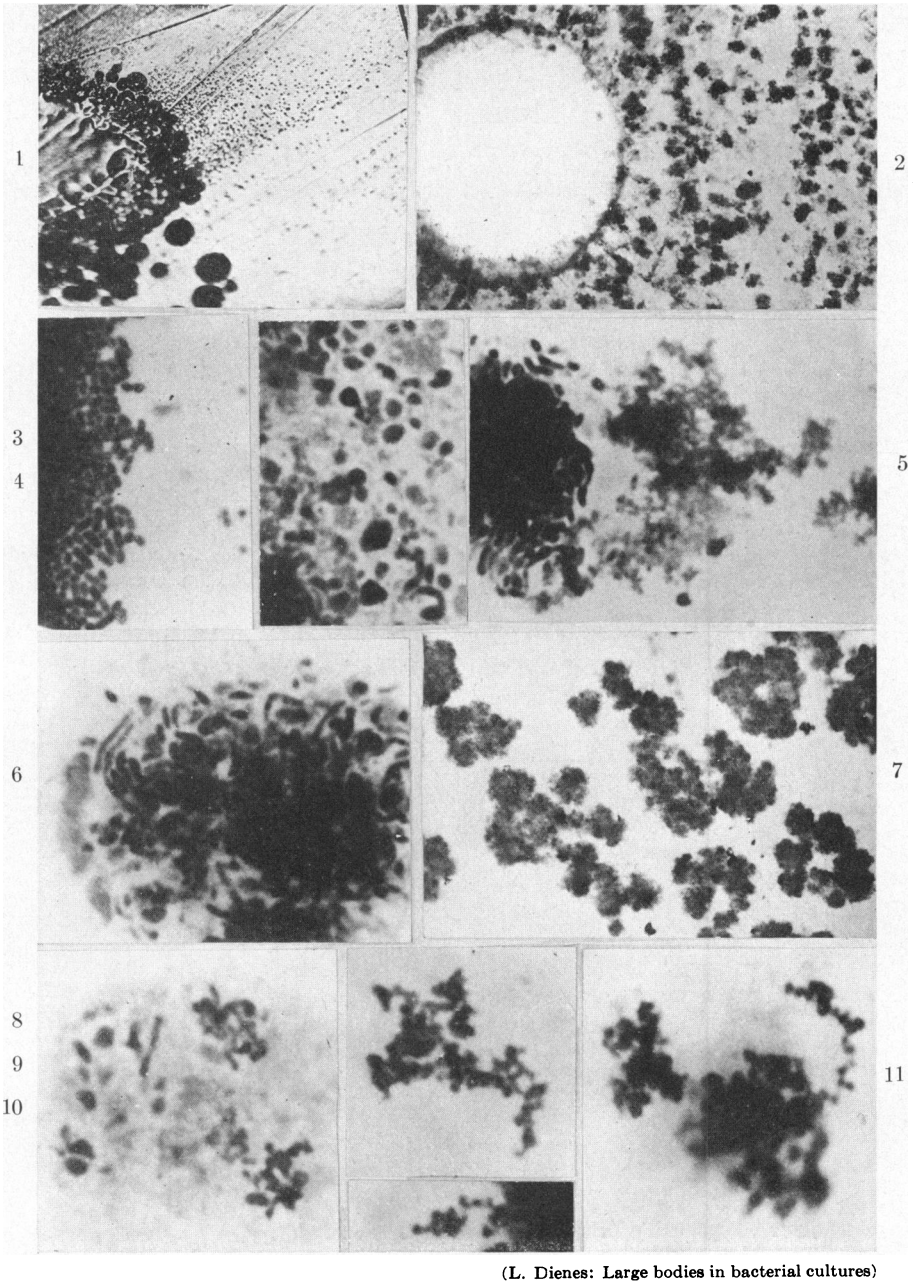
Photograph 9. A tiny L type of colony from Plate I. (× 3000.)

Photograph 10. Winding filament from the edge of an L type of colony (transplant from plate I). (× 3000.)

Photograph 11. L colony with high magnification. (× 3000.)

Photographs 8, 9, 10 and 11 were made from dry agar preparations.

Photographs 6 and 11 were previously published in a note (Dienes, 1939b).



(L. Dienes: Large bodies in bacterial cultures)

PLATE IV

Plate IV shows the different morphological forms observed in the cultures of colon bacillus strain 1706. All photographs were made from agar blocks stained with methylene blue and azur; 1, 2 and 4 were made from wet, the rest from dry preparations.

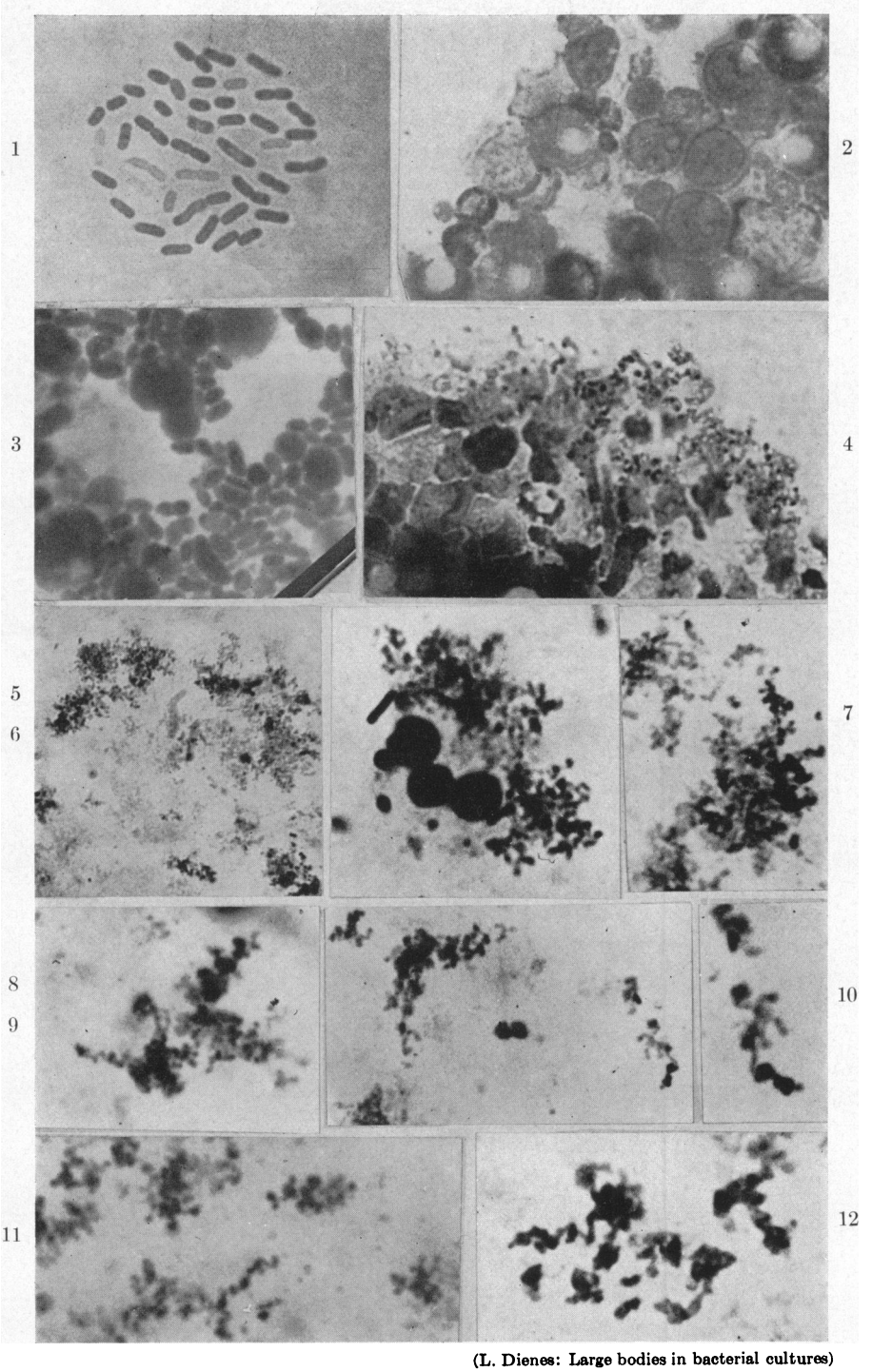
Photograph 1 shows a tiny colony (after 4-hour growth) photographed on the surface of agar. (× 1700.)

Photographs 2, 3 and 4 were made from colonies after about 24-hour growth. In 3 the culture was spread on the surface of agar during the process of staining. Transitional forms starting with small regular bacteria and continuing up to the large bodies are visible in the photograph. Photograph 2 represents the edge of a colony in which most of the bacteria are transformed into large bodies. Many of these bodies show one or more vacuoles. In 4 the large bodies at the edge of the colony are autolyzed and the elements of the developing L colonies are visible (× 1700). A comparison of photographs 2 and 3 shows how much more clearly visible the form and organization of the large bodies are in wet (photograph 2) than in dry preparations (photograph 3).

Photograph 5 shows the L type colonies in the agar after the bacterium colony has been washed away. (× 900.)

Photograph 6 shows a small autolyzed colony. A few large bodies and the L type of growth are visible in its place. The large bacillus visible in the photograph was carried over during the staining from a neighboring colony. (× 2000.)

Photographs 7–12 represent L type colonies developing under the bacterial colonies; 7, 8, 9 and 10 magnification (× 1700), 11 and 12 (× 2000). Photograph 10 represents the details of 9 with high magnification (× 3000). In 11 and 12 the pleomorphism and swelling up of the L form is visible.



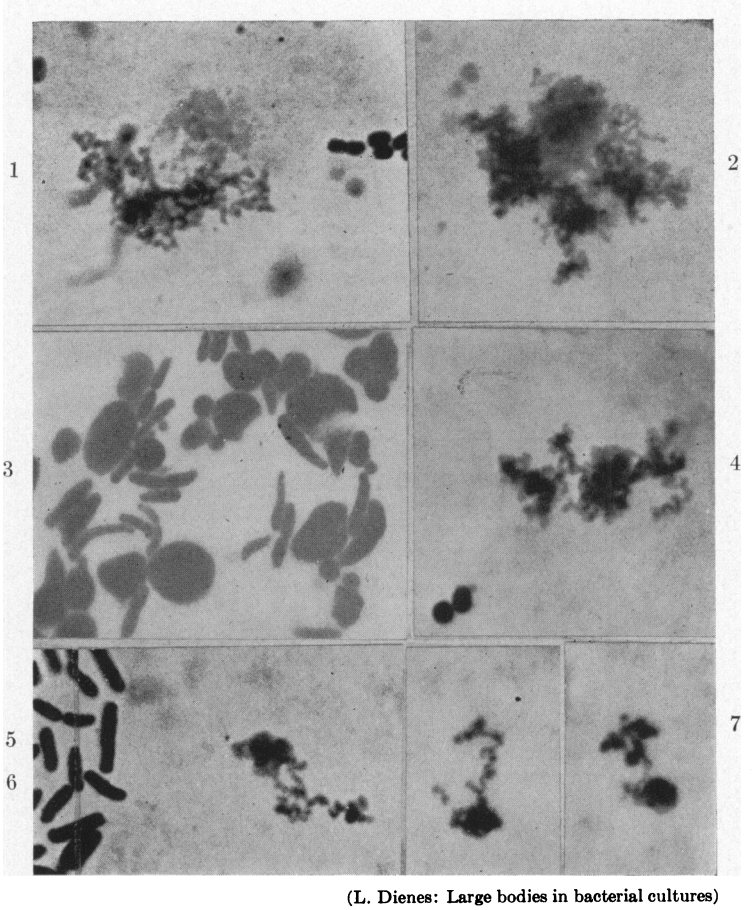
(L. Dienes: Large bodies in bacterial cultures)

PLATE V

The plate illustrates the germination of the large bodies in colon bacillus cultures Dry agar film stained with toluidin blue and thionin. (Magnification × 2000.)

Photograph 1 and 2 represents tiny colonies which developed from the large bodies of strain 1706 after 24 hour incubation.

Photographs 3–7 illustrate the growth starting from the large bodies of colon bacillus strain 12397. Photograph 4 represents about the largest size of the L type colony observed in the cultures of this strain. A part of the large body from which the colony developed is yet visible. Photograph 3 shows the gradual enlargement of bacteria into large bodies in strain 12395. In a large pear shaped body a vacuole is visible. The bacteria were spread on the agar surface during the staining.



(L. Dienes: Large bodies in bacterial cultures)