THE DEVELOPMENT OF PROTEUS CULTURES IN THE PRESENCE OF PENICILLIN[1,2]

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An interesting effect of penicillin was observed in agar plates containing the antibiotic and inoculated with *Streptobacillus moniliformis* and *Bacteroides* (Dienes, 1948*c*). The cultures of these species undergo a peculiar transformation under certain conditions. The bacilli swell into large round forms, the culture autolyzes, and a new growth appears that is similar in appearance and morphology to the pleuropneumonia group of organisms. Penicillin greatly favors this transformation. In the absence of penicillin only a few pleuropneumonialike colonies develop among the bacterial colonies; when penicillin is incorporated in the media, such colonies grow abundantly in pure culture. Some *Bacteroides* strains that do not produce pleuropneumonialike colonies without penicillin produce them abundantly on penicillin plates. It became apparent when the experiments were extended to other species that penicillin exerts a similar effect on many gram-negative bacilli. Pleuropneumonialike colonies have been isolated with the help of penicillin from *Proteus* (Dienes, 1948*a*), *Hemophilus influenzae* (Dienes, 1947), and *Eberthella typhosa* (Dienes, 1948*b*), from several *Salmonella* and *Shigella* strains, and also from gram-positive spore-bearing bacilli. Although they were not isolated in pure culture, their development was observed in many other species.

This peculiar effect of penicillin and the properties of the cultures observed in various species will be described in a series of papers. The observations made with *Proteus* are discussed first because they are more complete and certain aspects of the phenomena are more clearly apparent in them than in the other species.

The small bacillary forms of *Proteus* that are present in broth or in well-developed agar cultures under normal conditions of cultivation show no pleomorphism and no signs of development into L type colonies. These phenomena can be observed in the filamentous forms in the spreading zone of young agar cultures if normal growth is disturbed (Dienes, 1946). Many filaments in this zone are transformed under appropriate conditions into large bodies, which either return later to the bacillary form or grow into tiny pleuropneumonialike colonies. A tendency to become pleomorphic and to transform into L type of growth is present in *Proteus*, but it is much less apparent under normal conditions of cultivation than it is in *Streptobacillus moniliformis* or *Bacteroides*.

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This tendency as in the two other species is greatly increased by penicillin. Incorporated into agar or broth in concentrations of between 10 to 50 units per ml, penicillin inhibits the multiplication of most strains in the usual bacillary forms. On agar media of appropriate composition, growth continues in another form similar in morphology and growth properties to L_1 in *Streptobacillus moniliformis*. The bacilli swell into large round forms within a few hours, and small colonies develop during the following days on plates containing up to 10,000 units of penicillin per ml, the highest concentration that was tested. The development of these colonies has been variable in different experiments, and only after much experimentation were the conditions determined under which they developed regularly. The best results were obtained when fresh broth cultures were transferred to soft nutrient agar containing 10 per cent horse serum and the plates were incubated aerobically at 35 C. The medium was prepared by adding 50 to 100 per cent broth and 10 per cent red-colored horse serum, obtained by defibrination of the blood, to the tryptic digest agar used routinely in the laboratory. Serum obtained by coagulation of blood was unsatisfactory in most cases and was unimproved by the addition of laked horse blood. Penicillin is incorporated into the medium when the plates are poured. The morphology and the other properties of the cultures developing on these plates are very different from those of the usual bacteria. A detailed description is necessary to characterize them adequately. For this reason it is advisable to describe first in a general way the development of the cultures before describing the individual experiments in detail.

The first change apparent in plates containing 100 to 10,000 units of penicillin per ml is the development of a moist layer over the heavily inoculated areas. The slight haziness that remains on the plates after the broth culture is resorbed into the agar transforms in 6 to 14 hours into this moist layer. A few tiny colonies occasionally are apparent after overnight incubation. Numerous tiny colonies of uniform size and varying numbers of much larger colonies develop during the following days. Both types of colonies increase in size during the following 7 to 10 days. The appearance of a plate with well-developed colonies of both types is shown in photograph no. 1 of figure 2. This agar plate was lightly inoculated. Numbers 3 and 4 show two similar plates with the small and large colonies, respectively. The tiny colonies rarely develop to a size larger than 0.3 mm; the large type may grow to 2 mm or even more. The tiny colonies produce a confluent growth in heavily inoculated plates, but the large colonies usually do not coalesce. For reasons that will be apparent later, the tiny colonies are designated as colonies of stage 3A, the large ones as colonies of stage 3B.

The growth that develops in transplants made from these two types of colonies is markedly different. The large colonies of stage 3B reproduce the bacillary form of *Proteus* within a few hours if they are transferred to any of the usual liquid or solid media without penicillin. On soft horse serum agar containing penicillin abundant growth of the tiny stage 3A colonies develops. A few large colonies similar to the original growth also develop on some plates. This may occur in consecutive transfers if the large colonies are individually picked out

and transferred. In this way it is possible to keep them in continuous growth. Transplants to the usual hard agar plates usually produce no macroscopic growth. The growth of the large colonies in transfer was always scanty and even unobtainable with several strains. This behavior of the stage 3B colonies is surprising because they grow to a large size and their growth requirements, as will be seen later, are less exacting than those of stage 3A colonies. The large stage 3B colonies do not consist of penicillin-resistant bacilli, for not only is the morphology of the organism in the colonies changed, but they grow poorly on media containing either low or high concentrations of penicillin. Furthermore, the usual bacilli that return from the stage 3B colonies on penicillin-free media do not show increased resistance to penicillin.

The small colonies of stage 3A can be kept growing only on the soft horse serum agar plates. They grow equally well with or without penicillin. If penicillin is not present and the cultures are studied within a few weeks after isolation, the usual form of *Proteus* reappears and overgrows the plates in a few days. This does not occur in less than 24 hours, before the growth is well developed. It is possible to keep the cultures growing without reversion into the usual bacilli on penicillin-free media by daily transfer. If bacterial growth does not occur or is prevented by penicillin, the colonies continue to grow for 5 to 7 days. On heavily inoculated areas, an opaque, confluent, moist growth is produced that is firmly adherent to the medium. A few colonies may grow in old cultures to large size and take up the properties of stage 3B colonies. The return to the usual bacillary form in penicillin-free media is progressively delayed or may disappear completely if the stage 3A colonies are kept in cultivation in this form for several months. A pure culture of well-developed stage 3A colonies is shown in figure 2, no. 4.

The growth developing on penicillin plates was examined microscopically in unstained and stained agar preparations. A square of agar was cut out from the plates and covered with a cover slip. If staining of the culture was desired, a solution of methylene blue and azure was evaporated on the cover slip. The technique necessary for satisfactory staining has been described previously (Dienes, 1945). With this technique the whole growth developing on the plate, undisturbed in its original location, could be studied microscopically.

The bacilli transferred to plates containing sufficient amounts of penicillin (100 units or more) swell within 2 to 3 hours into large round bodies. This process is responsible for the appearance of a moist layer on the plates. The large bodies at first have regular contours and are deeply stained by methylene blue. If the broth cultures used for inoculation of the plates are young, all bacilli undergo swelling and no unchanged bacilli remain visible on the surface of the agar. Many bacilli are dead in old cultures, and their appearance is not affected by penicillin. During the following hours the large bodies continue to increase in size, their contours become irregular, and many disintegrate within 24 hours. A certain proportion of the large bodies, varying in different experiments, develop into tiny colonies by the growth of small round or bacillary granules. They are in groups and penetrate into the medium in various directions

from the large body. (The large bodies and the earliest stage of the L type colonies are illustrated in figure 3, nos. 2 and 3.) During the following days these tiny colonies develop into the macroscopical colonies described previously.

The characteristics of the stage 3A colonies will be discussed in some detail first. The appearance of the colonies in consecutive stages of development as seen with the low power of the microscope in unstained preparations is presented in the photographs. The highly refractile and granular appearance relates to the fact that the colonies are situated partly beneath the surface of the agar and do not present a smooth surface to the agar. The colonies of the organism of bovine pleuropneumonia in the photographs published by Ledingham (1933) have a similar appearance. The well-developed colonies consist of a dense spherical mass embedded in the agar. This is sometimes surrounded on the surface of the agar by a light periphery consisting of large round bodies. The periphery of the colonies may be wide (no. 7), narrow (no. 10), or absent. In the last case the center of the colony is well developed and is covered with large round bodies, which, however, spread only for a short distance or not at all on the agar. The dense center of the colonies consists of very small elements. No. 4 of figure 3 was made from an impression preparation and shows these forms varyingly swollen. The smallest elements appear as round corpuscles in the photograph. Often two or more small forms are in pairs or in short chains. Their size may range from 0.3 to 0.5 μ. At the edge of young colonies the small forms appear distinctly bacillary (figure 3, nos. 5, 6, and 7).

In preparations made with similar technique, the appearance of these small forms is entirely similar in *Proteus*, in the pleuropneumonia group, and in all the different bacterial species from which L type colonies were isolated. Their physical and staining properties are also similar in all cases. They are very soft and fragile. If a small piece of agar with the colonies is separated into fragments on a slide and then stained, the colonies are apparent in the agar, but the individual organisms are disfigured and usually cannot be recognized. The individual bacilli are clearly apparent when similar preparations are made from ordinary bacterial colonies. The morphology of stage 3A colonies is not influenced by the presence or absence of penicillin.

The growth properties of the stage 3A colonies are also similar to those of L_1. The colonies grow slowly and increase in size for many days. The colonies have the tendency to autolyze. They will reproduce the parent organism under appropriate conditions for a long period following isolation.

The large stage 3B colonies begin to develop like the stage 3A colonies. Their morphology and physical properties are also essentially similar. They are distinguished from the stage 3A colonies by their more vigorous growth, greater tendency to swell into large forms, more intense staining with methylene blue, and less pronounced autolysis. Large bodies begin to develop even in small colonies, not only on the surface but in the center embedded in the agar. The colonies show no sign of autolysis for several days. The center of the colonies contains small forms in dense groups, of the same type as those in stage 3A. These groups are surrounded by round forms that gradually increase in size.

These evidently originate by swelling of the small forms. A few small forms are always visible among the large ones. Double forms consisting of a large round body and a small granule are often seen. They do not represent budding but are the result of the uneven swelling of a pair. It is apparent at the extending edge of young colonies that the small forms multiply and penetrate the agar. The dense groups of small forms present in older colonies are apparently produced by disintegration of the large round forms and not by direct multiplication of the small forms. Among the small forms there are large round bodies filled with granules of similar shape and arrangement. This reproductive process is characteristic of the pleuropneumonia group (Klieneberger and Smiles, 1942). It explains the tendency of the small forms in the stage 3B colonies to swell and it is probably the main difference between the stage 3B and 3A colonies. In the latter, only the reproduction of the small forms is apparent. The large forms that develop on the surface of the colonies reproduce the small forms only if they are transferred to fresh media.

Efforts to make representative photographs of the small forms of stage 3B colonies and of the breaking up of the large bodies into the small forms were only partially successful. The organisms are too fragile to permit the use of smears. The small size of the organisms, their arrangement in pairs and short chains, and their gradual swelling are apparent in the photographs. For comparison, the small organisms from a pleuropneumonialike organism isolated from a human pharynx are shown in figure 3, no. 16.

When the stage 3B colonies are transplanted, growth starts exclusively from the large bodies. Their development can be followed without difficulty in slide cultures. Multiplication of the small forms is never visible in the transplants. This circumstance probably explains why it is difficult or impossible to propagate the stage 3B colonies in that form.

Colonies with properties similar to those of stage 3B were observed in addition to *Proteus* in *H. influenzae*. This type of colony usually develops alone on penicillin plates inoculated with *H. influenzae*. This occurs also in *Proteus* if the medium is not appropriate to support the growth of stage 3A colonies. Colonies similar in appearance to stage 3B were observed also in some *Salmonella* strains. The properties of these colonies have not been thoroughly studied.

In figure 1 the development of *Proteus* on penicillin plates is diagrammatically illustrated. Three consecutive stages are distinguished to facilitate the comparison of the behavior of different species and of the same species in different conditions. The first stage is the swelling of the bacilli into large bodies, the second the germination of these into tiny microscopical L type colonies. From this point on, development goes into a 3A stage, the growth of the small colonies, and into a 3B stage, the growth of the large type of macroscopical colonies. These consecutive stages follow one another in the cultures when L type colonies develop and are evidently parts of the same process. The development of the cultures may stop in any of these stages. For instance, in *Streptobacillus moniliformis* and *Bacteroides*, stages 1, 2, and 3 develop both in the absence and presence of penicillin. In several strains of *H. influenzae* and the colon bacillus,

stages 1 and 2 can be observed, but further development of the tiny L type colonies of stage 2 could not be induced. The germination of the large body produces a characteristic morphological structure that is sufficient to indicate transformation into the L form even if the latter does not develop to a further stage. Large bodies are produced from the bacteria under various conditions, and it is questionable whether they always indicate transformation into L forms.

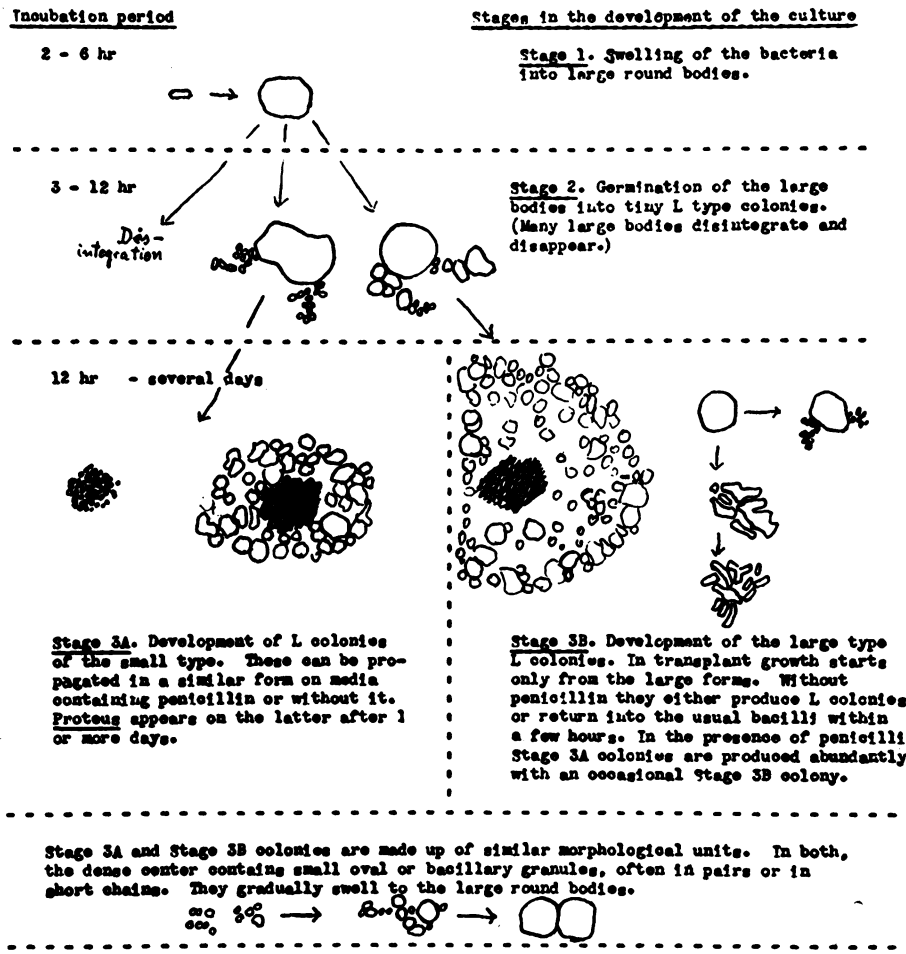

Figure 1. The development of *Proteus* on soft horse serum agar plates containing 100 to 10,000 units of penicillin per ml.

The description of the behavior of *Proteus* on penicillin plates is based on extensive experimentation. Between May, 1947, and June, 1948, forty experiments were made with 10 *Proteus* strains, using media of various composition and containing varying amounts of penicillin. It seemed worth while to concentrate on *Proteus* because the L colonies were easily obtained in this species and they reverted easily to the usual bacillary forms. The properties of the stage 3B

colonies appeared especially interesting. A few experiments will be described in some detail to illustrate the procedures and the actual observations.

Six *Proteus* strains (3, 26, 27, 31, 52, and 80), recently isolated in this laboratory, were inoculated on February 3, 1948, on soft agar plates containing 20 per cent horse serum and 100, 400, 1,600, and 5,000 units of penicillin per ml. Ordinary bacterial growth was inhibited in all plates. On the following day numerous small stage 3A colonies were visible in stained agar preparations in the cultures of every strain. They were smallest and least numerous in plates with 100 units per ml. In the cultures of strains 31 and 52, the earliest growth of stage 3B colonies became visible after 24 hours. The colonies increased considerably in size for several days and grew to the largest size in strain 52 with 5,000 units of penicillin. Stage 3B colonies of this strain developed abundantly on all plates. Fewer and smaller stage 3B colonies appeared in the other strains. The well-developed stage 3A and 3B colonies were similar to those illustrated in figure 2, no. 1.

Stage 3A colonies were isolated on February 13, 1948, from strains 3 and 26 and cultivated on penicillin plates until February 24. They were transferred five times during this period. From February 24 until March 8 they were passed six times on penicillin-free plates. The presence or absence of penicillin exerted no influence on their growth. The cultures remained free of *Proteus* in the first 24 hours, but, if they were kept for a few days, they were overgrown by *Proteus*. The same cultures were transferred again after 3 more months' cultivation on penicillin plates to penicillin-free agar and broth. The usual *Proteus* reappeared in these cultures after considerable delay, varying from 4 to 8 weeks. The tendency of stage 3A colonies to return to the usual bacillary forms decreases with prolonged cultivation just as the L_1 ceases to revert to the *Streptobacillus* type.

Between September and December, 1948, stage 3B colonies of strain 52 were passed in such form 16 times on soft horse serum agar plates containing 1,600 units of penicillin per ml. Single or small groups of well-developed stage 3B colonies were picked and transferred with the loop. Stage 3A colonies developed abundantly where the inoculation was heavy. On the areas lightly inoculated a few stage 3B colonies developed, never more than a few dozen. The colonies of the fifteenth passage, transferred to a routine blood agar plate, reproduced the usual bacilli within a few hours. The strain specificity of the recovered bacilli was unchanged and the resistance to penicillin was not increased.

The growth produced by stage 3B colonies transferred to penicillin-containing and penicillin-free media is illustrated in figure 2, nos. 14 and 15, after 24 and 4 hours' incubation, respectively. Many bacillary colonies developed within 4 hours when penicillin was not present. Only small stage 3A colonies developed in the presence of penicillin (no. 15). Several exposures were made at hourly intervals from the area illustrated in figure 2, no. 14. It is apparent in the photographs that the bacillary colonies developed exclusively from the large bodies.

The Conditions of Growth and Certain Properties of the L Type Colonies of Proteus

The development of stage 3A colonies and to a lesser degree of stage 3B colonies depends on the composition of the media. Cultures could be obtained and maintained in growth only on agar media and the consistency of the medium was of great importance. Only a few tiny stage 3A colonies develop with the usual content of agar (1½ to 2 per cent). The colonies autolyze and disappear in a short time. It has not been possible to keep these colonies growing on routine blood agar plates. Good growth is obtained by diluting the routine blood agar plates with equal amounts of broth. The colonies did not grow on coagulated blood serum.

The second important feature necessary for growth is the presence of animal serum. Many tiny colonies begin to grow in soft agar without serum, but their growth is limited and they soon die. Good growth is possible with 5 to 50 per cent horse serum, the optimum being around 10 per cent. It has already been mentioned that serum obtained by defibrination gave markedly better results than serum obtained by coagulation of the blood. Addition of red cells, whole or laked, did not significantly influence growth. Citrated human plasma, ascitic fluid, and rabbit serum gave good results in some experiments but were less uniformly satisfactory.

Stage 3A colonies did not develop on washed agar containing horse serum or on nutrient agar mixed with urine. If a piece of agar with young stage 3A colonies is transferred into horse serum broth, the colonies grow to large size on the agar, but growth so far has not been obtained in broth without agar.

Aerobic conditions favor growth more than anaerobic. Stage 3A colonies are often absent or rare on penicillin plates inoculated with *Proteus* and incubated anaerobically. In some cases the tiny stage 3A colonies developed a wide

Figure 2. Nos. 1 to 4 represent the growth of *Proteus* on penicillin plates. × 2.

1. The large and tiny colonies on a soft horse serum plate containing 5,000 units of penicillin per ml after 5 days' incubation.

2. Growth from the tiny colonies illustrated in no. 1 after several passages. The agar block used for transfer is visible.

3 and 4. Penicillin plates on which only the small (stage 3A) and the large (stage 3B) colonies developed.

5 and 6. Unstained cultures of stage 3A colonies. No. 5, one-day-old culture; no. 6, five-day-old culture. The irregular outline of the young colonies and the high refractility are characteristic and are in contrast to the bacillary colonies illustrated in no. 14. × 100.

7. Culture of a stage 3A colony having a typical deeply stained center and a wide peripheral zone. × 100.

8. Colony in no. 7 with higher magnification. Its surface consists of large round and irregular bodies some of which are vacuolated. Stained agar preparation. × 900.

9. Impression preparation from the same culture as that in no. 8 showing the periphery of a colony. × 2,000.

10. Stained culture of a stage 3A colony with a narrow peripheral zone. × 100.

11. The periphery of a colony from the same preparation as that in no. 10. × 2,000.

12. A stage 3B colony after 2 days' incubation. The relatively large size of the colony and the wide periphery consisting of large bodies are apparent. Unstained. × 100.

13. A similar colony from a stained agar preparation. × 900.

14. Small bacillary colonies after 4 hours' incubation on penicillin-free agar inoculated from stage 3B colonies. Unstained. × 100.

15. Small L type colonies on penicillin agar inoculated from stage 3B colonies. The colonies are much smaller than in no. 5, which represents a strain well adapted to the medium. × 100.

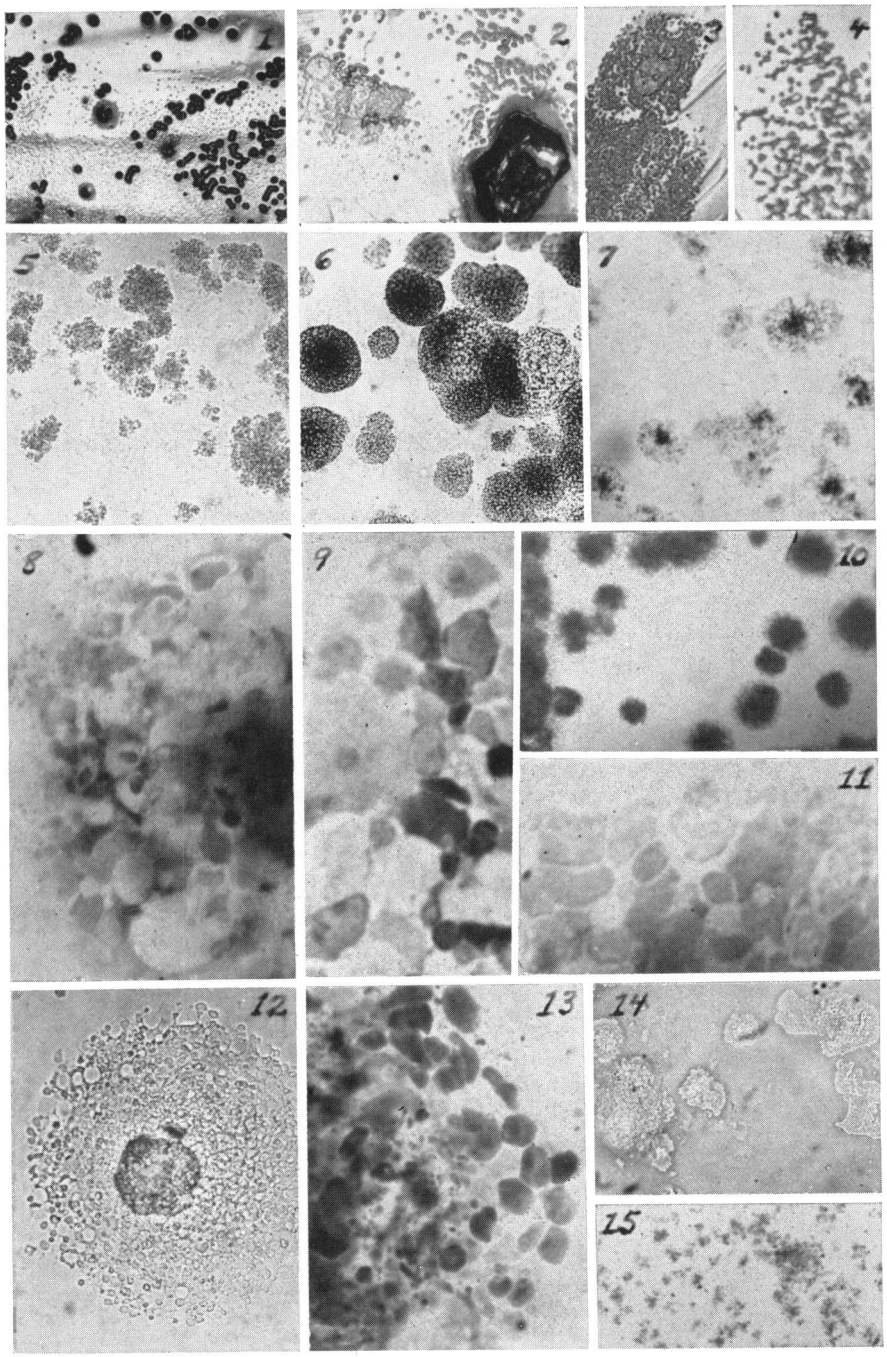

FIGURE 2

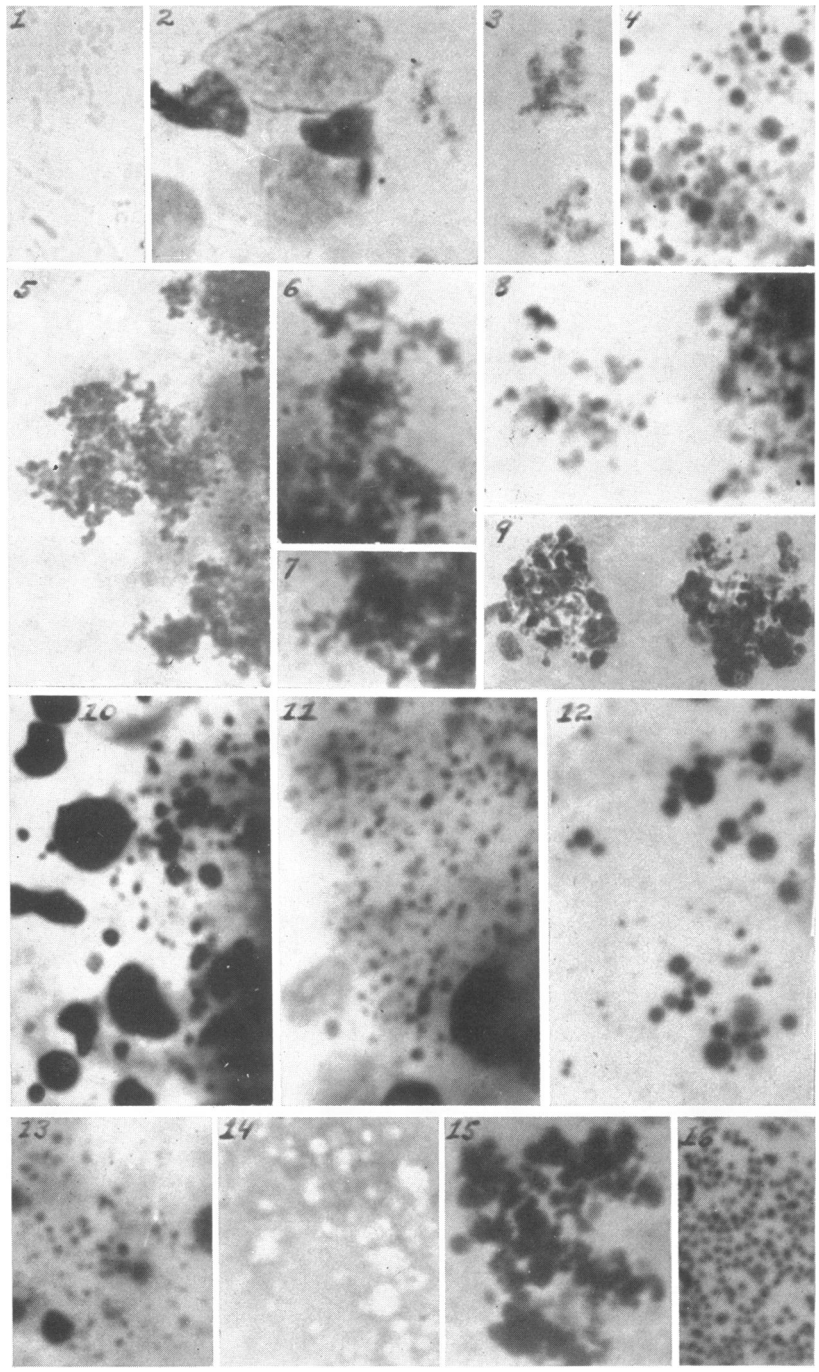

FIGURE 3

periphery on the surface of agar in anaerobic plates, and their appearance resembled closely that of the usual pleuropneumonialike colonies. Figure 2, no. 7, shows such a culture with low magnification.

The circumstances that determine the growth of stage 3B colonies are less well recognized. The conditions necessary for growth of stage 3A colonies exert no consistent influence. Sometimes they develop on hard agar plates without serum and sometimes, as in one of the experiments described, they are absent or rare on more appropriate plates. Apparently, great influence is exerted by the condition of the cultures used for inoculation of the plates. It is surprising that these colonies develop in large numbers and grow to considerable size on a plate, but when they are transferred to another area of the same plate and the medium is not appropriate for the growth of stage 3A colonies, usually no visible growth is produced. Both the stage 3A and 3B colonies develop to full size only if they invade the agar and a large part of the growth is beneath the surface of the medium. This property is common to the L_1 and the whole pleuropneumonialike group. It is probably a consequence of this property that growth is usually

Figure 3. The morphology of the organisms in stage 3A and stage 3B colonies illustrated with high magnification.

1. Bacilli on a penicillin agar plate immediately after inoculation. Unstained. × 2,000.
2. Same plate as in no. 1 after 8 hours' incubation. All bacilli swelled to large forms and a tiny L type colony begins to grow. Stained agar preparation. × 2,000.
3. Another field from the negative of no. 2 illustrating two tiny L type colonies. These are embedded tridimensionally in the agar and only a small portion of the colonies is in sharp focus. A few very small bacillary forms are visible. Stained agar preparation. × 2,000.
4. Impression preparation from well-developed stage 3A colonies after agar fixation. The surface layer of the colonies was washed off in order to obtain a preparation from the deeper layer. Stained with thionin. × 3,000.
5. Fresh secondary growth at the edge of a large autolyzed stage 3A colony. The growth is under the surface of the agar and the individual organisms and their arrangement in the spreading cultures are fairly visible. Dry stained agar preparation. × 2,000.
6 and 7. The spreading edge of young stage 3A colonies. Dry stained agar. × 3,000.
8. A very small stage 3A colony and the edge of a larger one. Dry stained agar. × 3,000.
9. Two small stage 3A colonies. Very small and large swollen forms are present. Dry stained agar. × 900.
10, 11, 12, and 13. These photographs were made from stage 3B colonies with the same technique as that used in no. 4. The surface of the colonies was scraped off to obtain an impression from the center of the colonies. The agar blocks were fixed on the cover slip and the preparations stained with thionin. The small forms are often in pairs or short chains. Gradual transition from the smallest to the largest forms is apparent in nos. 10 and 12. Double forms consisting of organisms of different size are often seen. The smallest elements visible in the photograph are about 0.3 microns. × 3,000.
14. Dark background preparation made with nigrosin from stage 3B colonies. The surface of the colonies was scraped off and a block of agar containing the colonies was placed on a cover slip. A small drop of nigrosin solution was placed beside the block, which covered the impression left by the colonies when the agar block was lifted. The purpose of these preparations was to ascertain whether the stained structures represented the whole organism. × 3,000.
15. A young stage 3B colony in a stained agar preparation. It is apparent that the colony extends by a growth similar to that of stage 3A colonies, although the individual elements have a great tendency to swell into large forms. × 3,000.
16. Impression preparation obtained from a pleuropneumonialike organism isolated from human sputum. Although the individual organisms are more clearly visible in this photograph, their size and arrangement are similar to those of the small forms in stage 3B colonies. Nile blue. × 3,000.

excellent on pieces of agar submerged in broth, but, with most strains, growth is slight in liquid media. It is remarkable that although the growth requirements of stage 3B colonies are simple, these colonies were observed only on agar media. No similar growth develops on coagulated serum or in broth. The growth into agar is apparently not an accidental property of pleuropneumonialike organisms, but is the consequence of some essential requirement for their growth.

The growth of the colonies of stage 3A is markedly favored by crowding. The colonies grew to the largest size in a crowded area, and their size decreased near the edge of the inoculated area. Single colonies far from the others usually stop growing and autolyze. The effect of crowding on the growth of the colonies is apparent in figure 2, no. 2. A similar effect of crowding is present in the L colonies isolated from a *Flavobacterium* and gives a peculiar appearance to the cultures of L forms in these two species. No similar effects of crowding were observed in L cultures isolated from other bacteria and in the pleuropneumonia group.

The *Proteus* bacilli swell to large forms in broth to which penicillin has been added as they do in agar. The large bodies persist in such forms in the broth, but they do not multiply and a growth comparable to stage 3A and 3B colonies does not occur. The large bodies remain visible for a long time and, transferred to agar, grow either into L type colonies or return to the usual bacillary forms. Broth cultures kept for 4 days with 1,600 units of penicillin per ml, transferred to agar, reproduced within a few hours the *Proteus* bacilli.

Seven *Proteus* strains isolated in this laboratory and examined shortly after isolation all produced stage 3A and 3B colonies under appropriate conditions. There were consistent differences between the strains. Some produced one, some the other, type of colonies more abundantly. In many experiments strains 3 and 26 produced few or none of the stage 3B colonies, but these were usually abundant with strains 14 and 52. No stage 3A or 3B colonies could be isolated from the *Proteus* strains X_2 and X_{19}, which had been kept in artificial cultivation for many years. Tiny 3A colonies started to develop abundantly, but they could not be grown on subculture. Stage 3B colonies developed in some experiments with X_K, and the stage 3A colonies of this strain could be adapted to continuous growth. Long artificial cultivation also affects the viability of L type colonies in typhoid and *Salmonella* strains. L type colonies could be isolated and kept in cultivation only from freshly isolated strains.

The serological properties and the metabolic activity of the L strains isolated from *Proteus* have been studied to a certain extent. Agglutinating sera were prepared in rabbits with the bacilli and the L strains were isolated from them. Sera obtained with either culture agglutinated both in high titer in the same way as the sera obtained with L_1 and *Streptobacillus moniliformis* agglutinate both organisms. Thus, the antigen specificity of the L form and of the parent organism is very similar. Serological tests with the L form present certain technical difficulties, and the procedures used require a detailed description. This and the protocols of the serological tests will be presented in a subsequent paper.

The most characteristic metabolic activity of *Proteus* is urea fermentation.

This could be demonstrated also in the L cultures. These cultures grew well on agar diluted with an equal amount of urine to which 10 per cent horse serum had been added. They produced a strong ammoniacal odor and volatile alkali on such media. The L cultures did not show another characteristic of *Proteus* cultures, the digestion and liquefaction of proteins. This was tested by growing the cultures on serum agar plates in which the serum was partly coagulated by heat. No clearing of the turbidity occurred around the colonies.

Development of L Type Colonies in the Absence of Penicillin and the Nature of the Influence of Penicillin

It has been mentioned that the spreading filaments of *Proteus* are transformed into large bodies if they are exposed to slight injuries of various types, and occasionally these grow into tiny L colonies (Dienes, 1946). Some of the injuries that produce this effect are physical, such as refrigeration; others are disturbances in the environment, such as transfer into tap water or ascitic fluid. The most pronounced effect was produced by the antagonism between spreaders belonging to different strains. The spreading filaments of one strain do not penetrate a territory covered by filaments of another strain. They are transformed in the contact zone, if the strains are appropriate, into large round bodies that break up later either into small bacilli or develop into tiny L colonies. The phenomena observed in the contact zone of different strains are similar in many respects both to the spontaneous autolysis that in many species precedes the growth of L type colonies and to the phenomena produced by penicillin. They are probably produced in all these cases by the presence of antagonistic substances interfering with normal growth.

It is of interest that only the spreading filaments are susceptible to the influence just mentioned. The small bacilli present in broth culture or on agar before and after the spreading of the culture are not affected by them. The condition of the culture is of primary importance for the transformation into L forms.

It is necessary to indicate briefly the evidence for regarding the tiny microscopical L type colonies developing from the spreading filaments as essentially similar to the L colonies isolated with the help of penicillin. The morphology of these colonies is similar if they are compared in the corresponding stage of development. Large bodies are produced from the filaments in the contact zone of two strains and in the presence of penicillin by "plasmoptysis." If the large bodies are cultivated in the absence of penicillin, they develop in both cases either into bacillary or L type colonies. It is unlikely that such complex processes of transformation observed in the same species and similar in every detail represent unrelated phenomena. Thus far, it has not been possible to isolate the tiny colonies from the bacilli in the absence of penicillin, but, as far as their development could be followed, it was similar to that of the colonies induced by penicillin.

Changes occurring in various bacterial cultures in which L colonies develop spontaneously suggest also that this process is a response to influences interfering with usual growth (Dienes, 1942; Dienes and Smith, 1944). L colonies develop in most cases after multiplication stops and the cultures autolyze. This may

occur in young cultures after a few hours' growth. Examples of such early autolysis were observed in *Streptobacillus moniliformis*, *Flavobacterium*, and *Bacteroides*. The morphological changes in the bacteria preceding the growth of L type colonies are similar whether these colonies develop following autolysis or following exposure to penicillin.

Bacteroides strain 132, which was used for various studies, offered an opportunity to compare the effects of autolysis and penicillin (Dienes, 1948*c*). It was available both in cultures that autolyzed spontaneously and those that lost this property. In the latter, penicillin produced morphological changes similar to those that developed spontaneously in the autolytic cultures. Various observations suggest that autolysis of the cultures is the consequence of the diffusion of antagonistic substances through the media. The morphological changes that indicate autolysis appear almost simultaneously in all bacilli in broth and on agar in large areas of the plates. It is especially significant that the bacteria in the process of change return to the normal shape and resume normal multiplication immediately if they are transferred to fresh media.

According to these considerations, the growth of L type colonies is induced by similar influences either when they develop spontaneously or under the influence of penicillin. The only difference is that in spontaneous autolysis the substances interfering with normal growth are produced by the bacteria themselves. This supposition agrees with the fact that penicillin induces growth of L type colonies in many species, whereas they develop spontaneously only in those in which autolysis is observed. For instance, spontaneous development of L colonies was never observed in freshly isolated typhoid bacilli and *Salmonella*, in which autolysis is rare, although such colonies are regularly produced in penicillin plates. Autolytic strains are frequent in *Bacteroides*, and these often produce L type colonies spontaneously.

According to these considerations, the influence of penicillin in inducing transformation of bacteria into L type colonies probably is not specific. The bacteria may react in a similar manner to various influences, interfering with normal growth. It is obscure at present why some interferences produce and some others do not produce this effect.

Following the discovery of this effect of penicillin, influences of various types were studied to determine whether they induce growth of L colonies. Many gram-positive and gram-negative bacteria were exposed to heat and cold, variation of pH, and bacteriostatic substances of different types. Media of varied composition were used in the experiments. The results of the experiments were for the most part negative with the exception of one chemical that had an effect similar to that of penicillin, though of lesser degree.

The first group of substances studied were the antibiotics streptomycin, citrinin, aspergillic acid, and tyrothricin. They inhibited bacterial growth but produced no morphological changes and no L colonies. Under the conditions in which they were tested, they also inhibited the growth of L_1 and of the pleuropneumonialike organisms isolated from patients. The second group of substances included sulfa drugs, phenol, formaldehyde, and mustard gas. The results with these were also negative. Lithium, cadmium, and mercury salts in

appropriate concentrations produced large bodies from various bacteria, but these did not develop into L colonies either on the original media or on transfer to media without the salts.

The chemical that induced the growth of L colonies was carboxylmethoxylamine. The bacteriostatic effects of this drug were studied by Favour (1948), and a sample of the drug was received from him. The growth of *Proteus* was partially inhibited by 0.05 and 0.10 per cent and completely by 0.20 per cent of this drug. The bacilli swell into large bodies on soft horse serum plates containing the lower doses, and many of them developed during the following hours into tiny L colonies. These increased considerably in size overnight, but their isolation was not possible because the plates were overgrown by the spreading *Proteus*. Larger doses of the drug inhibited all growth and the bacilli remained unchanged in form on the surface of the plates. The margin between the doses inhibiting bacillary growth and those producing L type colonies is very narrow with this drug.

Carboxylmethoxylamine exerts a similar influence on the cultures of *H. influenzae* and *S. moniliformis*. The zone in which bacterial growth is inhibited and L type of colonies are produced is also narrower in these species with this drug than with penicillin.

The success in producing L type colonies with one chemical seems to be more significant that the failures to which reference was made. It lends further support to the conclusion that the effect of penicillin is not specific.

DISCUSSION

The observations with *Proteus* suggest certain considerations concerning the nature of L type colonies. The first is the similarity of the processes observed in different bacterial species regardless of whether the L colonies develop spontaneously or whether they are produced by penicillin. The morphology of these colonies and the successive steps through which the usual bacillary forms are transformed into the L forms are also similar. This process develops in conditions disturbing or inhibiting the normal growth of bacteria. This may occur spontaneously as a result of autolytic processes, or it can be induced by chemicals such as penicillin and carboxylmethoxylamine, and in some cases by physical influences such as exposure to cold. The whole process, the transformation of the bacterial cell and growth in a new form, is apparently a specialized reaction of the bacteria that can be elicited under various conditions in many species.

Considering the observations made with *Proteus*, there remains little doubt that the bacillus and L organisms are growth forms of the same organism. In the stage 3B colonies not only is their derivation from the bacteria apparent, but the return of bacterial forms from the large bodies formed in these colonies also can be observed directly in slide preparations. The stage 3A colonies do not return so easily into the usual bacilli. This process occurs if the cultures are allowed to age on penicillin-free media. Repeated subcultures on plates containing high concentrations of penicillin and the fact that the bacilli recovered from L

colonies are not resistant to penicillin eliminate the possibility that the L cultures carry accidentally included bacilli. Although the L forms are morphologically different from the bacilli and also their growth requirements are different, they are similar to the bacilli in serological properties, and they also possess one of the distinctive metabolic characteristics of *Proteus*, urea fermentation. The usual bacilli were regained from the L cultures so far in three genera: *Proteus*, *Streptobacillus moniliformis*, and *Bacteroides*. The conditions under which these observations were made exclude the persistence of bacilli in the L cultures as a contamination. It is increasingly difficult to maintain Klieneberger-Nobel's supposition (1948) that the L forms are symbionts or parasites in the bacterial cultures.

The significance of the L form in the life of bacteria is not known. These forms are more resistant to certain injuries than the bacteria. The three groups that were most thoroughly studied, *Streptobacillus moniliformis*, *Bacteroides*, and *Proteus*, survive and multiply in penicillin plates in the L form and, when penicillin is eliminated, may return to the usual bacillary form. The cultures behave in a similar way in spontaneous autolysis. This resistance may be an essential property of the L forms or it may accidentally accompany their unknown real functions.

It was mentioned in a previous paper that transformation into L forms often is preceded by the bacteria taking on the characteristics of rough growth (Dienes, 1942). This is apparent also with penicillin. Concentrations of penicillin that just permit growth induce the bacteria to grow in long filaments and rough colonies. Somewhat higher concentrations induce transformation into large round bodies. Such observations suggest that the growth in L colonies may be the end stage in the smooth to rough transformation. No changes occur in the genetic characteristics of the strains when they are recovered from the large bodies either directly or following passage through the L forms.

The properties of stage 3A and 3B colonies give some suggestions concerning their relationship. Stage 3B may represent the fully developed variant. Stage 3A may be an imperfect development of the former. The growth requirements of stage 3B are less exacting than those of stage 3A and are nearer to those of the usual bacilli. The stage 3B colonies grow vigorously and return easily to the usual bacilli. The organisms seem to persist and grow in this form under certain types of adverse conditions that inhibit growth in the usual bacillary form. The delicate growth requirement of stage 3A colonies and their increasing difficulty in regaining the usual bacillary form would make them less useful as resistance forms. They may represent the variant in an unsuccessful form that is kept alive only by the artificial conditions created in the cultures. It is of great interest that in the stage 3B colonies the reproduction of the small forms inside the swollen forms is well established. The essence of the transformation into L forms may be the substitution of one type of reproductive process for another. This interpretation of the nature of L type colonies and of the differences between the stage 3A and 3B is entirely hypothetical, and it is offered with reservations like the suggestion made previously that the L colonies may be similar to the "haplo-

form yeast" of Winge. It is equally possible that the stage 3A colonies represent the end of the transformation and that the stage 3B is an intermediary stage nearer to the usual bacillary forms. The frequent occurrence of cultures with properties similar to those of stage 3A on the mucous membranes of men and animals suggests adaptation of these cultures to special conditions. The fact that the L type colonies are produced under conditions inhibiting the usual bacterial growth rather adds than detracts from the interest attached to them, even if we have no clear idea of their nature.

SUMMARY

Tiny pleuropneumonialike colonies that were previously described in *Proteus* were grown with the help of penicillin to larger size and could be maintained indefinitely in subculture. The appropriate composition of the medium is of great importance for the growth of these colonies.

Multiplication of most freshly isolated *Proteus* strains is inhibited in the usual bacillary forms by the addition of 10 to 50 units of penicillin per ml of the medium. However, on soft horse serum agar plates in the course of a few days colonies develop in media containing up to 10,000 units of penicillin per ml. They grow in two distinct types. One type remains small on the original plate (0.05 to 0.2 mm) and grows easily in transplants whether or not penicillin is present. If penicillin is absent, the usual bacillary form of *Proteus* reappears within one or a few days or up to several weeks, according to the time that has elapsed since the isolation of the culture. The colonies of the second type are larger (1 to 3 mm). Transferred to penicillin-free media, they reproduce the usual bacillary forms within a few hours. On media containing penicillin, they produce an abundant growth of the small colonies with an occasional large colony. Both the small and the large type of colonies are similar in morphology and physical properties to the L_1 developing in *Streptobacillus moniliformis*. The bacilli recovered from these colonies have no increased resistance to penicillin and are not changed in their genetic properties. The large colonies are evidently a growth form of *Proteus* because they are derived from the bacilli and return easily to this form. That the small colonies are also a growth form of the bacillus is evidenced by their reversion to the usual bacillary form and by their similarity to the bacilli in serological properties and metabolism.

Proteus, like *Streptobacillus moniliformis* and *Bacteroides*, survives and multiplies in the L form under appropriate conditions on agar plates containing high concentrations of penicillin. When L colonies develop spontaneously, their role apparently is similar. The cultures undergo autolysis, the bacillary forms are destroyed, and growth continues in the L form. Transformation into this form is apparently a response to certain types of adverse conditions and is not a specific effect of penicillin.

The effects of various types of bacteriostatic chemicals were studied. One of them, carboxymethoxylamine, had an effect similar to that of penicillin and induced growth of L colonies. The effect was quantitatively much less than that of penicillin.

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