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Reproductive Processes in Proteus Cultures.*

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A different and more complex form of reproduction than binary fission has been observed in Gram-negative bacteria.[1] In exceptional strains of several species most of the bacteria swell up into large round bodies which in turn develop into regular bacteria or a peculiar variant form. Sometimes bacteria form inside the large bodies; sometimes the latter disintegrate into irregular fragments which after a few divisions assume the usual bacterial form. The peculiar variant form consists of small soft granules which have a tendency to invade agar and to swell into large round bodies. This variant is similar in appearance and properties to the pleuropneumonia group of organisms. Following Klieneberger, it is designated with the letter L. Klieneberger[2] discovered this type of colony (L_1) in cultures of Streptobacillus moniliformis. The L_1 variant isolated from this species is easily reversed to its parent form during a varying length of time after isolation. It is serologically similar to the parent form. The L_1 appears to be an intermediary link in the development of bacteria from the large bodies.[3] The reversion of L forms into the parent bacterial forms has not been observed in other species. The complexity of these reproductive processes and their similarity in different species makes it unlikely that they are degenerative. It has been pointed out elsewhere[3] that the L forms present similarities to certain yeast forms which Winge[4] obtained by cultivation of single spores of an *ascus* and which he designated "haploform."

All bacteria in which these processes have been observed were isolated from pathological processes or from mucous membranes. On artificial media the bacteria were not in their natural environment. For this reason it was thought that a study of saprophytic organisms, to which artificial media are not so foreign, might afford some information as to the significance of these reproductive processes. The study of Proteus was undertaken because large bodies are present in small numbers in the spreading halos of most strains. There is some uncertainty as to whether the Proteus strains used in the present study are truly saprophytic because they were isolated from urine and stool specimens. However, Proteus does not seem to be adapted to a true parasitic life, and its natural habitat is outside the living organism.

The Proteus cultures develop in consecutive phases of growth *in situ,* and of spreading. The bacteria are markedly different during these phases. The growth starts in a lightly inoculated plate as small bacilli with no tendency to spread. After the colonies reach a certain stage of development the bacilli at the edge of the colonies grow into filaments abundantly provided with flagellae and start to move actively on the surface of the medium. These actively moving filaments produce the spreading halo. When the halo reaches a certain size the filaments break into short rods and multiply as such without further spreading. The filaments appear again after a certain interval, and the spreading is resumed. Filaments usually are not produced in liquid media. Of 30 strains studied, 25 grew in spreading cultures; 5 strains lost this characteristic soon after isolation.

Accidental observations suggested that large bodies are more numerous in mixed cultures of different strains. Experiments made to study this point brought unexpected results.

When broth cultures of certain strains are

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1 Dienes, M., and Smith, W. E., *J. Bacteriology,* 1944, **48**, 125.

2 Klieneberger, E., *J. Path. Bact.,* 1935, **40**, 93.

3 Dienes, L., to be published.

4 Winge, O., *Compte. r. Trav. Lab., Carlsberg,* 1935, **25**, 72.

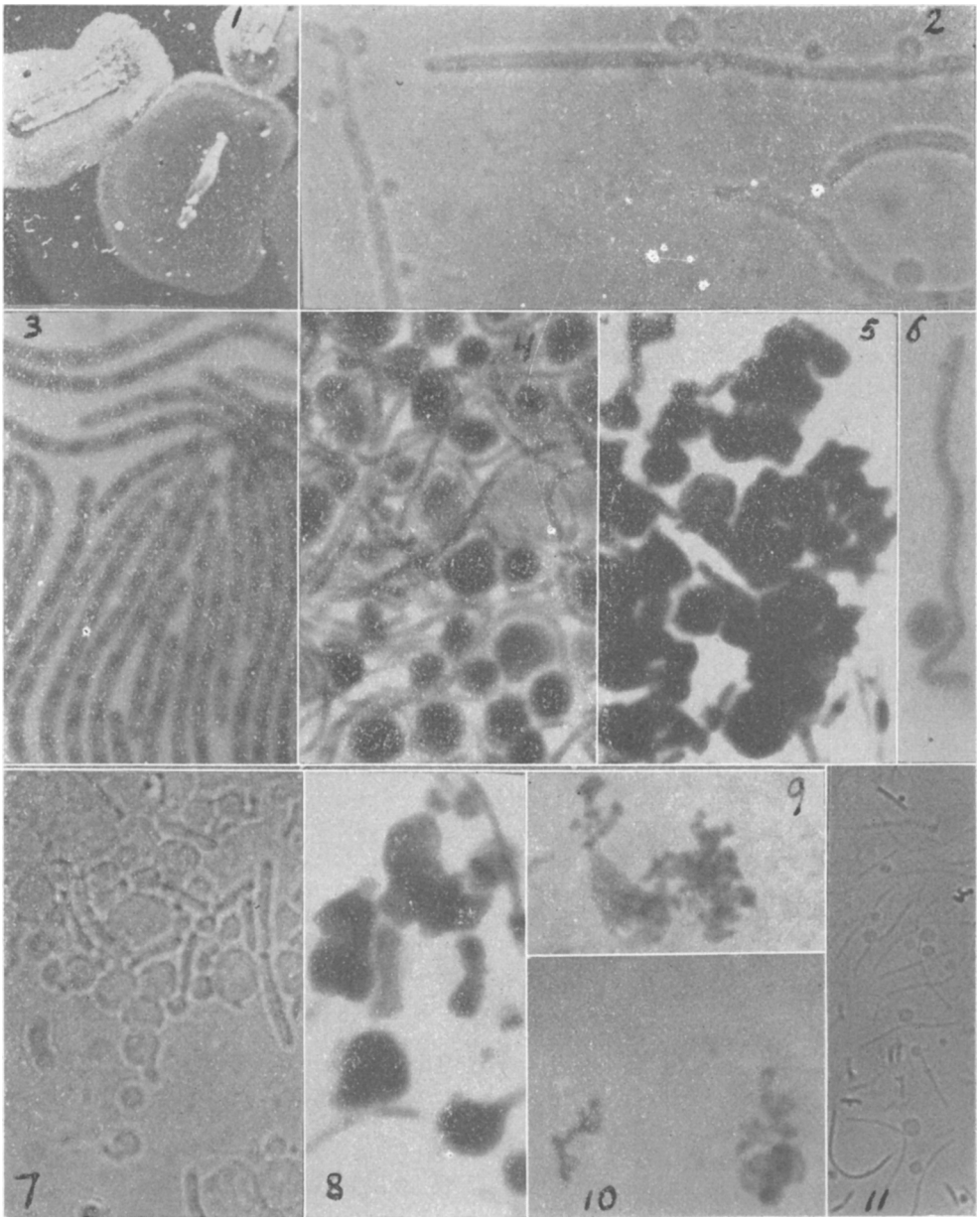

Fig. 1.

Photograph 1. The meeting of spreading halos of strains 3 and 14 on a blood agar plate. Note the line of demarcation between the halos.

2. Filaments of strain 3 transferred to distilled water. Their content oozes out as droplets attached to the filaments. Unstained, × 3000.

3. Spreading filaments of a halo. Wet agar preparation stained with methylene blue. × 3000.

4. Large bodies at the meeting line of 2 halos. Most of the filaments are attached to the large bodies and are shrunken. Dry impression preparation after agar fixation stained with methylene blue. × 3000.

5. Large bodies on the surface of agar inoculated with the spreading filaments of 2 halos. Incubation, about one hour. The deformation of large bodies is the first sign of their development into usual bacterial forms. Only few bacterial filaments remained visible between the large bodies. Giemsa staining after agar fixation. × 3000.

6. A bacterial filament at the meeting site of 2 halos with a large and a tiny round body attached to it. Wet agar preparation stained with methylene blue. × 3000.

7. Large bodies developing on agar inoculated with filaments from 2 halos. Only few bacteria retained their usual shape. Unstained, × 2000.

8. Large bodies enlarged and deformed from a culture similar to that represented in Photograph 5. Lighter staining with methylene blue allows better recognition of the shape of the large bodies. × 3000.

9 and 10. L type of growth originating from large bodies produced in tapwater and transferred to agar. The large bodies are deformed and stain unevenly. In the left lower corner of Photograph 10, the small bacillary and round forms of the L type of growth are fairly well visible. Dry agar preparation stained with methylene blue and azur. × 3000.

11. Spreading filaments transferred to distilled water. Nearly all filaments produced large bodies. Unstained. Moderate magnification, × 1000.

mixed and planted on agar, large bodies appear in clusters situated at the edge of and between the originally developed colonies when the spreading starts. The large bodies are attached to the bacterial filaments (Photographs 2 and 6, Fig. 1). The interaction between strains can be observed most clearly when strains are planted separately on agar and the zone where the spreading halos meet is examined. It is usually necessary to examine several oil immersion fields in a spreading halo to find one large body. On the other hand when 2 halos meet, most of the filaments are transformed into large bodies, and are thus immobilized within a short time (10 to 30 minutes). Photographs 3 and 4 illustrate the organisms in a halo and in the meeting zone of 2 halos, respectively. In Photograph 3 there are long, winding filaments which appear segmented when stained with methylene blue. In Photograph 4 most of the organisms are in the form of large bodies. The visible filaments are shrunken and in most cases are attached to large bodies. Large bodies are also abundantly produced when agar blocks containing the halo of one strain are cut out and superimposed on the halos of another strain.

The production of large bodies in mixed cultures depends on 2 fundamental conditions: the strains must be appropriate, and spreading halo must meet spreading halo.

Filaments spreading from 2 colonies of the same strain exert no influence on each other when the halos meet. Two of the 30 strains studied (3 and 7) produced large bodies with all the others. They did not influence each other. The next best strain was tested with 12 others and produced large bodies with 8, including strains 3 and 7. The number of strains examined is not sufficient to indicate whether there are groups of strains which react with other groups or whether some strains are more apt to produce large bodies than others.

The meeting of the cultures produces no effect if one of them is in the small bacillary form, the stage preceding spreading. Only motile filaments are transformed into large bodies and only when they meet motile filaments of another strain. Whether the filaments of both strains are involved cannot be determined with certainty. When the filaments of one strain are shorter than those of the other, large bodies are attached both to the longer and to the shorter filaments. This observation suggests the participation of both strains. The fact that at certain sites nearly all filaments have large bodies attached to them also suggests that both strains are involved.

The mixing of strains is not the only way to induce the transformation of spreading filaments into large bodies. Exposure of the culture to cold or transfer into tap or distilled water or into broth containing small amounts of $HgCl_2$ often induce this transformation. If the cultures containing fresh halos are kept overnight in the refrigerator, most filaments develop into large bodies within a short time after the cultures are warmed to room temperature. Large bodies appear also within a few minutes if the spreading filaments of some strains, especially those of strains 3 and 7, are transferred into tapwater. The mechanism by which the large bodies are produced can be most clearly observed in tapwater. They are produced by a process which has long been known as "plasmoptysis." The contents of the filaments ooze out and remain attached to the filaments as small droplets. A droplet may

increase rapidly in size and may develop within 1 or 2 minutes into a full-sized round body. Meanwhile the filament shrivels. It is thus apparent that most of the content of the filaments flows out to produce a large body. Large bodies are produced in a similar way in mixed cultures or after refrigeration. During this process, although the filaments shrink visibly, they retain their motility both in tapwater and on the surface of agar and drag their attached large bodies rapidly about among other bacteria. The large bodies are bent and deformed by collisions; they may be drawn through narrow passages. It is therefore apparent that the large body is a resistant elastic structure firmly attached to the filament. It very rarely happens that a large body is destroyed by mechanical injury. In this case it disappears without leaving a trace.

In tapwater, filaments of different strains produce large bodies in varying proportions. In strains 3 and 7 many filaments are transformed into them. In most strains only a few large bodies are so produced. Transfer from agar into broth or ascitic fluid also induces the production of a few large bodies. The small bacilli of the first growth stage are not affected by exposure to cold or to tapwater.

Most filaments of strain 3, when transferred into broth containing 0.01% $HgCl_2$ were transformed into large bodies, but this concentration prevented further growth. When the concentration of $HgCl_2$ was 0.005 and 0.0025% the small bacilli remained motile, continued to multiply and many filaments produced large bodies. They were produced, as in tapwater, by "plasmoptysis" · $HgCl_2$, many other toxic salts and penicillin also induce the formation of large bodies in various other bacterial species. Phenol has no similar effect. Large bodies produced from Proteus or other bacteria under these conditions are not viable.

The viability of large bodies and the type of growth which they produce varies according to the circumstances under which they are produced. Most of the large bodies produced in mixed cultures and after refrigeration will grow and change back within a short time to the usual small bacteria. This process occurs in a manner similar to that observed in Bacteroides.[1] The large bodies increase in size and their contour becomes irregular. They fractionate and after further growth and division their parts regain normal bacterial form. Photographs 5 and 7 show the enlarged irregular bodies which represent the first step in the retransformation into bacteria. It is apparent in the picture that nearly all large bodies are developing. At certain sites hardly any filaments survive, and the new growth originates from large bodies. At first growth is retarded at the meeting of spreading halos. This retardation is temporary, and the large bodies soon resume multiplication. The development of tiny bacterial colonies from large bodies has been observed in slide cultures. It seems unnecessary to publish a photographic record of this process because this has already been done in the case of Bacteroides. Occasionally, as in other species, L type colonies develop from these large bodies.

The development of large bodies produced in tapwater is not uniform. Most of them do not develop when transferred to agar. A few may produce the L type growth. The morphology of the parent form is produced only on rare occasions. Tiny L type colonies growing from large bodies are illustrated in Photographs 9 and 10. They reach their highest development after 8-12 hours' incubation, and rarely grow to larger size than the colony shown in Photograph 8. They are covered by bacterial growth originating from filaments not transformed into large bodies and disappear. Their isolation in pure culture has not been successful. The L type growth in Proteus is very similar to the corresponding growth in *E. coli*.[5]

The differences in the development of the large bodies produced in mixed cultures and after exposure to refrigeration on one hand, and in tapwater and in broth containing $HgCl_2$ on the other hand are so marked that there must be important differences between the large bodies themselves. It has already been mentioned that large bodies produced by $HgCl_2$ did not develop when transplanted. The author previously has observed that the

[5] Dienes, L., *J. Bacteriology*, 1942, **44**, 37.

large bodies produced in cultures of various bacteria are viable only when they appear during the natural development of the cultures. When toxic influences induce the formation of large bodies, they probably awaken a latent tendency in the bacteria in an imperfect manner, but they do not produce viable large bodies and are not able to bring the process to its normal conclusion. The difference between the large bodies produced by different means in Proteus is the more remarkable because they seem to be produced in mixed cultures by dissolved antagonistic substances just as they are in a medium containing $HgCl_2$ or penicillin. It was not possible to substantiate the natural first impression that large bodies developing in mixed cultures are sexual forms. Conjugation is not apparent in mixed cultures either between filaments or between large bodies. No other formal elements which could indicate a sexual process were visible in preparations stained with Giemsa or with flagellar-staining methods. On the other hand, the antagonistic effect of the strains on each other is apparent. Halos starting from colonies of different Proteus strains do not penetrate into each other. Even after several days of incubation a sharp line of demarcation persists between the areas covered by different strains. The halos sometimes stop at a short distance from each other without coming into contact, indicating the diffusion of antagonistic substances into the medium. Meanwhile extension proceeds in other directions. Large bodies are not produced without contact between filaments, but this fact could be explained by differences in concentration of the antagonistic substances. The present observations suggest that large bodies are produced in mixed cultures by dissolved substances and that viability distinguishes such large bodies from those produced by toxic influences.

There are observations which suggest as a possibility that the influence of one strain on the other may be more than a simple antagonism. Avery's[6] observations on the interchange of capsules between pneumococcus strains suggest that hereditary characteristics in bacteria can be transmitted by dissolved nuclear substances without morphological organization. If this is true, processes corresponding to sexuality in higher organisms may be possible in bacteria without an exchange of organized nuclei. The fact that in Proteus 2 strains are involved in the production of large bodies presents an opportunity to study whether an exchange or redistribution of hereditary characters occurs during this process.

Summary. The complex reproductive processes formerly observed in certain Gram-negative bacteria have also been observed in Proteus cultures. In this species they presented several distinctive characteristics. The large bodies in every case previously studied were produced by gradual swelling of the bacteria. In Proteus they are produced by a process known as "plasmoptysis." The large bodies so produced possess similar physical properties and show reproductive processes similar to those formed in other species by swelling of the bacteria.

Another characteristic of Proteus is that the bacteria from which the large bodies develop differ considerably from the bacteria usually present in the culture. Large bodies develop only from spreading filaments actively motile on solid media. In almost every culture these filaments produce an occasional large body, and most of them are transformed into large bodies when the spreading halo of one strain meets the spreading halo of another appropriate strain. The large bodies so produced are viable and within a short time are transformed into regular bacteria. Exceptionally they produce the L type of growth. There is a great variation in the reaction of different strains to each other.

The spreading filaments show a tendency to "plasmoptysis" and production of large bodies under various adverse conditions, for example, when they are subjected to refrigeration or when they are transferred into tapwater or into broth containing $HgCl_2$. The large bodies produced by refrigeration are apparently fully viable. Those produced by tapwater are only occasionally viable, and when they germinate they usually produce L type colonies. Large bodies produced by

[6] Avery, O. T., MacLeod, C. M., and McCarthy, M., *J. Exp. Med.*, 1944, **79**, 137.

$HgCl_2$ are not viable. The difference in viability constitutes an important difference between the large bodies produced by the interaction of the 2 strains and by refrigeration on one hand, and by toxic influences on the other hand. The meeting of the 2 strains induces a complex reproductive process consisting of several consecutive steps. After a short delay vigorous multiplication is resumed. It is not likely that this process is simply degenerative.

No union was observed between cells at the meeting of 2 strains and the present observations do not support the supposition that the interaction between the strains is sexual.

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Differences in Culturability, Infectivity and Pathogenicity of Human Strains of *Endamoeba histolytica.**

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Introduction. Clinical and experimental data have provided a wealth of divergent views concerning the ability of *Endamoeba histolytica* to produce disease. In man and experimental animals there is a wide range of clinical states in amebiasis, from a fulminating infection to an essentially symptomless condition. Both the intrinsic pathogenicity of the particular "strain" of the organism and the host's general threshold of resistance to infection contribute to infectability and the degree of pathology produced.

Since the accumulated knowledge fails to explain many theoretical and practical questions concerning *E. histolytica* and its host, a renewed attack has been made on the problem. The particular objective of the present study consists in an evaluation of culturability, infectivity and pathogenicity of different "strains" of the parasite.

Material and Technics. Eighteen persons have served as sources of the parasite. The youngest was 2 years and the oldest 59 years old. Twelve were males, 5 females and the record on one was not available. Six were white, 12 were Negroes and one was an Indian. All but one, a Honduran, were American-born and the majority were natives of Louisiana who had never left the state. Twelve persons sought medical help because of colitis, including diarrhea and chronic dysentery. Two individuals in the series were suffering from bronchopneumonia. One patient complained of rheumatism and one was suffering from oxyuriasis. A food handler undergoing routine coprological diagnosis had no complaint. The clinical diagnosis of one was not obtained. Of the entire group 7 passed formed stools from which cysts of *E. histolytica* were obtained and 4 submitted unformed stools containing motile trophozoites of the organism. In 5 instances proctoscopic material was obtained for inoculation. In one case a purgative specimen was used. In another, a dysenteric stool was cultured in the hospital laboratory. In 11 instances cysts of trophozoites in freshly passed stools were utilized.

The basic culture medium consisted of an egg-slant base, made of a coagulated emulsion of whole egg and Ringer's solution, with liquid overlay of physiological salt solution containing 0.5% crude liver extract (Lilly). After tubing, a small amount of specially milled rice starch, furnished by Doctor Charles W. Rees of the National Institute of Health, Bethesda, Md., was sterilized and planted in the overlay. Formed stool specimens provided cysts, which were concentrated by the zinc sulfate centrifugal floatation technic, thoroughly washed in distilled water and then seeded in the tubes. Small amounts of mucus

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