NUTRITION OF CELLULAR SLIME MOLDS

I. Growth on Living and Dead Bacteria

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Received for publication 15 August 1962

ABSTRACT

HOHL, HANS-RUDOLF (University of Wisconsin, Madison) AND KENNETH B. RAPER. Nutrition of cullular slime molds. I. Growth on living and dead bacteria. J. Bacteriol. **85**:191–198. 1963.—Methods for growing selected species of cellular slime molds in liquid culture on living and dead bacteria are described. Species investigated included *Polysphondylium pallidum*, *P. violaceum*, *Dictyostelium discoideum*, and *D. purpureum*. Maximal growth of myxamoebae occurred in suspensions of 10^{10} living bacteria (*Escherichia coli* B/r)/ml in Sörensen's phosphate buffer (pH 6.0), reaching a density of 10^7 to 2×10^7 cells/ml in 48 hr. The generation time for the different slime molds ranged from 2.4 hr for *P. violaceum* to 2.9 hr for *D. discoideum* (strain V-12). Good growth of *P. pallidum* occurred between pH 3.6 and 7.8. The slime molds grew less well on dead (autoclaved) than on living bacteria and, except for *P. pallidum*, the amount and rate of growth decreased markedly as the time of autoclaving was increased from 2.5 to 80 min. Bacteria killed with propylene oxide supported growth equal to those autoclaved for a few minutes. The myxamoebae were very sensitive to the osmotic pressure of the culture medium, especially in the presence of living bacteria, and addition of as little as 0.01 M NaCl caused a measurable decrease in slime mold growth. The culture techniques employed afford useful methods for investigating the nutritional requirements of the cellular slime molds, and the experiments described provide the bases for subsequent studies relating to the axenic cultivation of these singular microorganisms.

Despite many attempts, the problem of growing cellular slime molds in pure culture without bacteria or bacterial products has remained unsolved. Whereas cell aggregation, pseudoplasmodial migration (if present), and culmination of the cellular slime molds proceed in the absence of any bacteria, vegetative growth and multiplication of the myxamoebae rests on the engulfment of bacterial cells.

Refuting earlier claims of an intimate association (even symbiosis) between these slime molds and the accompanying bacteria, Raper (1937) and Raper and Smith (1939) showed that *Dictyostelium discoideum* could be grown in two-membered culture with a wide variety of species, including gram-negative and gram-positive types and forms pathogenic to animals and plants as well as saprophytes. Raper further demonstrated that bacteria killed by heat or ultraviolet radiation still supported the growth of the myxamoebae. Bradley and Sussman (1952) and Sussman and Bradley (1954) extracted from certain gram-negative bacteria a proteinaceous factor that permitted limited growth of *D. discoideum* when this extract was applied as a paste on the surface of a specially constituted agar base. The difficulties of preparation and the relatively low yields of myxamoebae obtained have precluded its further use (Sussman, 1961). Attempts to grow cellular slime molds in the absence of bacteria or bacterial components have been unsuccessful up to this time (Raper, 1937; Heller, 1947; Hirschberg, 1950).

Agar-solidified media in petri dishes were used in all of the aforementioned investigations. In the present study we have taken advantage of the submerged-culture technique developed by Gerisch (1959, 1960). This technique has the advantage that all myxamoebae grow under the same conditions; that quantitative measurements can be performed easily; and that the sterility of the medium can be tested more accurately.

This report deals with the technical details of our test system and with experiments relating to the growth of certain slime molds, especially *Polysphondylium pallidum* and *P. violaceum*, on

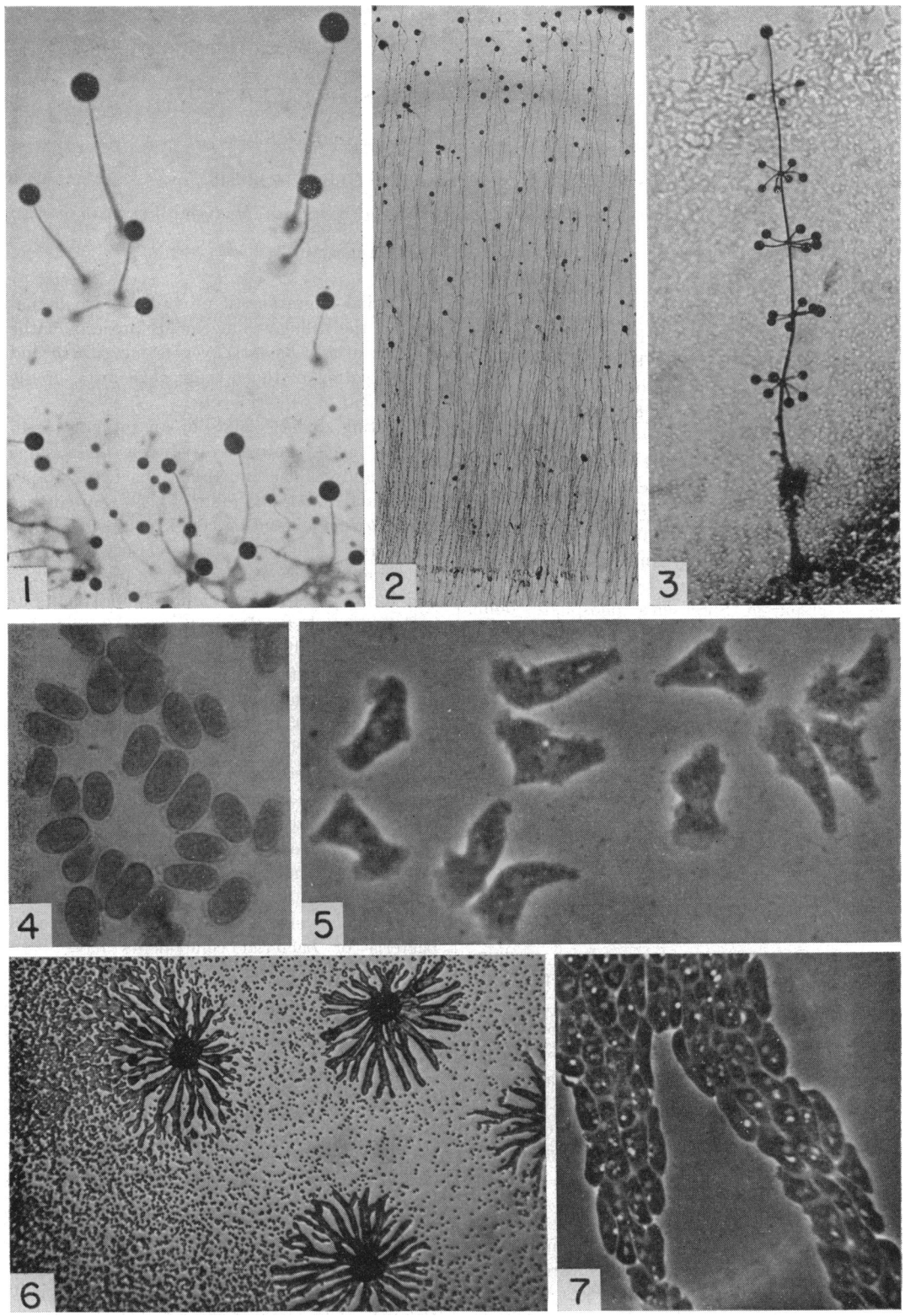

FIG. 1–7.

living and dead bacteria. In the paper that follows (Hohl and Raper, 1963), the growth of *P. pallidum* in axenic culture is described.

Materials and Methods

Primary emphasis was placed on two strains of *Polysphondylium*, namely, *P. pallidum* WS-320 and *P. violaceum* P-6; *D. purpureum* WS-321 and *D. discoideum* strains NC-4 (type) and V-12 were included in several experiments. Fructifications, or sorocarps, of three of these cultures are illustrated in Fig. 1 to 3, whereas stages in the development of a single strain, *P. pallidum* WS-320, are shown in Fig. 4 to 7. The bacterium used as nutrient was *Escherichia coli* B/r, a culture obtained from Günther Gerisch, University of Freiburg, Germany. For the usual procedure, *E. coli* was pregrown in 500-ml Erlenmeyer flasks, each containing 100 ml of the following medium: Tryptose (Difco), 5 g; yeast extract (Difco), 5 g; dextrose, 1 g; $K_2HPO_4 \cdot 3H_2O$, 1 g; and tap water to 1,000 ml. The pH was adjusted to 7.0. The inoculated flasks were incubated on a rotary shaker at 30 C. After 19 to 24 hr of incubation, the bacteria, usually reaching a concentration of 5 or 6 $\times$ 10^9 cells/ml, were transferred aseptically into sterilized tubes and centrifuged on a Servall medium angle centrifuge, Type S.P., for 15 min at 900 $\times$ *g*. The bacteria were washed twice with half the original volume of Sörensen's phosphate buffer (0.016 M; pH 6.0). Following this, the bacteria were resuspended in the buffer and their concentration adjusted to 10^{10} cells/ml.

Two different culture methods, both modifications of Gerisch's (1959) technique, were adopted. (i) Samples (5 ml) of bacterial suspension were dispensed into test tubes (18 by 150 mm), inoculated with the slime mold, and incubated at 25 C on a rotary shaker at 250 rev/min. (ii) Quantities (100 ml) of bacterial suspension in 500-ml wide-neck tissue-culture bottles were similarly inoculated. Each bottle was closed by a rubber stopper from which a small Teflon-covered magnet was suspended and through which ports for aeration and sampling were provided. Incubation was at 25 C on a magnetic stirrer, with a piece of cardboard between the stirrer and bottle to prevent overheating. Cultures were inoculated with either spores from young sorocarps (1 to 7 days) to give a concentration of 2 $\times$ 10^3 spores/ml of medium, or vegetative myxamoebae, pregrown on autoclaved bacteria in a tube culture to give a concentration of 10^4 myxamoebae/ml.

To ensure the absence of living bacteria in the inocula and the autoclaved bacterial suspension, controls were made by inoculating tubes of nutrient broth. In some cases, agar plates were employed as additional controls. The experiments with dead bacteria were performed using bacterial suspensions autoclaved for 20 min at 121 C if not otherwise stated. The experiments were done in triplicate. The myxamoebae were counted with a hemacytometer, and the average of four or more counts per series was calculated. The concentration of the bacteria was determined turbidimetrically using a Bausch & Lomb nephelometer and a standard curve; pH was measured with a Beckman G pH meter.

Results

Growth on living bacteria. The growth of *P. pallidum* WS-320 in a stirred flask culture is shown in Fig. 8. The inoculum was at a level of 2 $\times$ 10^3 spores/ml of medium. After a lag phase of 12 hr, the number of myxamoebae increased exponentially for about 27 hr, with a generation time of 2.6 hr. After 47 hr, the myxamoebae had completed their growth and reached a final concentration of about 1.3 $\times$ 10^7 cells/ml. When myxamoebae were used as inoculum, the lag phase was reduced to 8 hr.

Essentially the same results were obtained

FIG. 1. *Mature sorocarps of Dictyostelium discoideum NC-4 grown in association with Escherichia coli on an agar plate.* $\times$ *9.0.*

FIG. 2. *Dictyostelium purpureum WS-321 grown in association with Escherichia coli on an agar plate illuminated from one side, hence the very long sorophores.* $\times$ *1.8.*

FIG. 3 to 7. *Polysphondylium pallidum WS-320 grown in association with Escherichia coli on agar plates. (Fig. 3) Mature sorocarp showing its characteristic branched habit.* $\times$ *15.0. (Fig. 4) Mature spores.* $\times$ *1,400 (stained). (Fig. 5) Myxamoebae at the close of the vegetative phase and preceding cell aggregation.* $\times$ *800 (phase contrast). (Fig. 6) Wheel-like pseudoplasmodia formed by inflowing streams of aggregating cells.* $\times$ *18. (Fig. 7) Detail of two small, converging streams showing uniform orientation of the myxamoebae.* $\times$ *500 (phase contrast).*

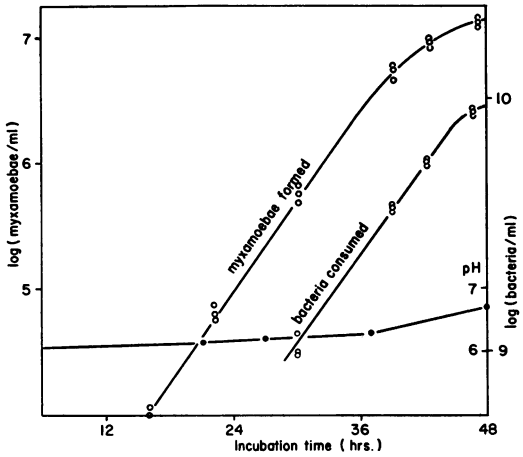

FIG. 8. *Cell growth, consumption of living bacteria, and pH of the medium in triplicate cultures of Polysphondylium pallidum* WS-320.

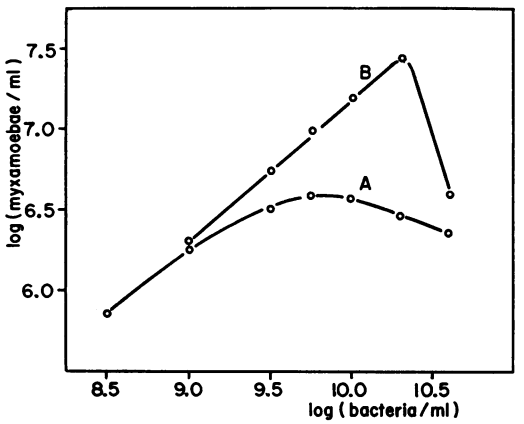

FIG. 9. *Relative growth rate* (A) *and final cell concentration* (B) *of Polysphondylium pallidum WS-320 growing in media containing different densities of living bacteria.*

when other slime molds, such as *P. violaceum* P-6 or *D. purpureum* WS-321, were grown under similar conditions (Fig. 12). The results are also comparable to those obtained by Gerisch (1959, 1960) with *D. discoideum* V-12, but the generation times of all strains tested in our studies were shorter than the 3.3 hr for strain V-12 reported by Gerisch. We obtained generation times of 2.6 hr for *P. pallidum* WS-320, 2.4 hr for *P. violaceum* P-6, 2.6 hr for *D. discoideum* NC-4, 2.4 hr for *D. purpureum* WS-321, and 2.9 hr for *D. discoideum* V-12. *D. discoideum* was grown exclusively in shaken tubes since it often showed irregular growth in stirred flask cultures. The myxamoebae of all species grown in submerged culture fruited normally when removed and placed on non-nutrient agar in petri plates.

The influence of the concentration of nutritive bacteria on the growth rate and final concentration of the myxamoebae was tested with *P. pallidum* WS-320. The results are given in Fig. 9. Curve A shows the concentration of the myxamoebae at the end of the log phase, and hence represents the relative growth rate of the myxamoebae; curve B gives the final concentration obtained. It may be seen that the optimal rate of growth and the highest final concentration of myxamoebae occurred at different bacterial concentrations.

P. pallidum WS-320 was grown at different pH levels, ranging from 3.7 to 7.8. Very good growth occurred with 0.016 M Sörensen phosphate buffer within its effective range (6.0 to 7.8). The growth curves of pH 6.0 and 7.0 were almost identical; in contrast, the onset of growth at pH 7.8 was delayed for about 15 hr, but afterward the myxamoebae grew at almost optimal speed. This prolongation of the lag phase is probably due to the effect of high pH on spore germination (Raper, 1939). The growth rate of *P. pallidum* WS-320 in McIlvaine's buffer (0.1 M citric acid, 0.2 M Na_2HPO_4) is usually less than in Sörensen's buffer. Slight changes in concentration of the former have a marked effect, and best results were obtained when it was used at one-eighth strength. At a pH below 4.5, the bacteria tended to clump together, and at pH 3.6 they settled out and had to be resuspended by stirring. Nevertheless, the spores germinated within the whole range tested (pH 3.6 to 7.8), and except for a delay of ca. 5 hr the myxamoebae multiplied at pH 3.6 and 4.2 almost as fast as in phosphate buffer at pH 6.0, which is optimal. In contrast, between pH 4.5 and 7.8 the slime mold was markedly inhibited in McIlvaine buffer, and its growth was far less than with phosphate buffer of the same pH. A discrepancy of a similar kind was observed by Takeuchi and Tazawa (1955) when measuring the influence of pH on pseudoplasmodium formation in *D. discoideum*. With both buffer systems, the pH tended towards a final value of 6.8 to 7.2.

Difficulties in growing bacteria and *P. pallidum* WS-320 together in a Tryptose-glucose-yeast extract broth (Sussman, 1961) led us to evaluate the influence of the osmotic pressure of the

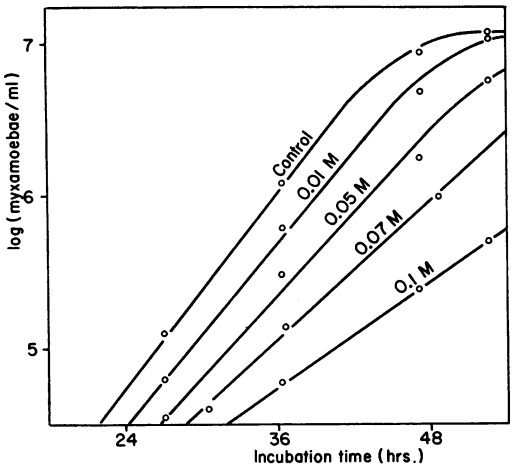

FIG. 10. *Influence of NaCl concentration (0 to 0.1 M) on the growth of Polysphondylium pallidum WS-320 in a suspension of living bacteria.*

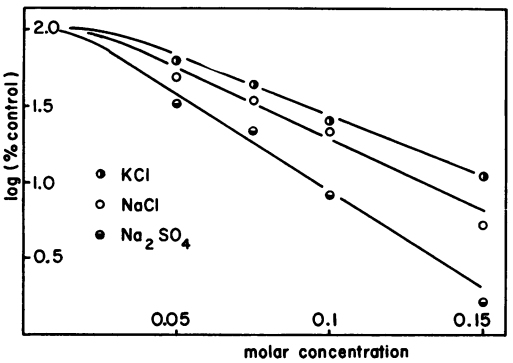

FIG. 11. *Relative growth of Polysphondylium pallidum WS-320 in a suspension of autoclaved bacteria containing varying concentrations of NaCl, KCl, and Na_2SO_4.*

medium. In the first experiment we added different concentrations of NaCl to the bacterial suspension, and, after inoculation with 2×10^3 spores/ml of medium, the growth of the myxamoebae in the stirred flasks was measured. Relatively low salt concentrations diminished the rate of growth (Fig. 10). Further experiments on osmotic pressure were done with autoclaved bacteria in tube cultures. To eliminate a possible influence on spore germination, the inoculum consisted of myxamoebae pregrown on autoclaved bacteria. Figure 11 summarizes the influence of three salts on cell growth after a period of 45 hr, as compared to the control without additional salts. A depression of growth to about one-third [log (% control) = 1.5] occurred with a concentration of 0.08 M NaCl. With a suspension of living instead of dead bacteria, 0.01 M NaCl brings about the same reduction (Fig. 10). Na_2SO_4 was most effective in depressing growth, and sucrose (not included in Fig. 11) was least effective. The order of activity could be correlated with the degree of dissociation of the compounds tested. Essentially the same results were obtained with *P. violaceum* P-6, yet the latter strain was slightly less sensitive to increased osmotic pressure.

Growth on dead bacteria. The usually difficult task of obtaining uncontaminated inocula for pure cultures (e.g., Daniel and Rusch, 1961) is minimized in some species of the cellular slime molds because the spore-bearing sori are in most cases already free from contamination. A control experiment showed 100% of the sori of *P. pallidum* WS-320 and 87% of those of *P. violaceum* P-6 to be free of bacteria. If desired, penicillin may be added to the cultures to preclude bacterial growth; 50 units/ml proved to be slightly stimulatory for the growth of *P. pallidum* WS-320 but to have no effect on *P. violaceum* P-6. The antibiotic nystatin, a fungal inhibitor, decreased slightly the growth of both strains at a concentration of 20 μg/ml.

Growth curves for three slime molds tested on autoclaved bacteria, compared to their growth on

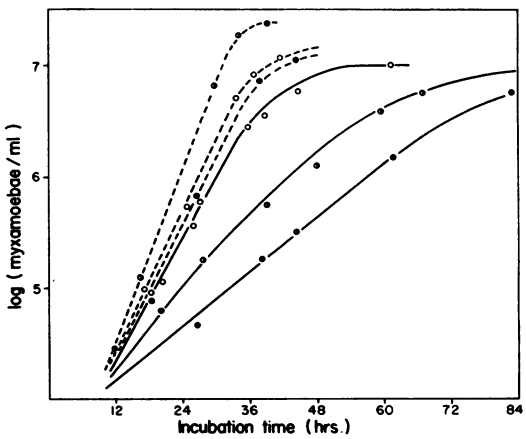

FIG. 12. *Growth of Polysphondylium pallidum WS-320* (○), *P. violaceum P-6* (◑), *and Dictyostelium discoideum NC-4* (●) *in suspensions of autoclaved bacteria (solid lines) compared to their growth on living bacteria (dotted lines).*

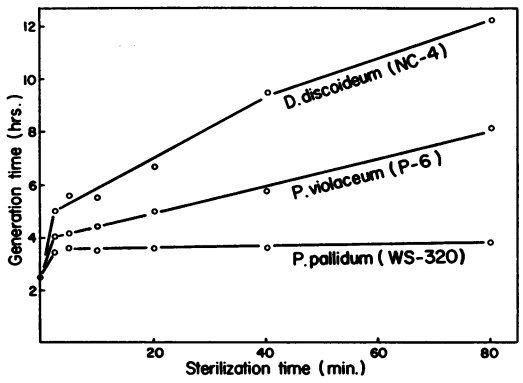

FIG. 13. *Generation time of different slime molds growing in suspensions of bacteria autoclaved for periods of time ranging from 2.5 to 80 min.*

living bacteria, are presented in Fig. 12. Essentially the same curves were obtained with both stirred flask and tube cultures. Growth was roughly exponential in all strains tested, but it sometimes tended to be slightly faster in the beginning as seen for *P. violaceum*. Also, killed bacteria were usually consumed less completely than living bacteria, resulting in a slightly decreased final concentration of myxamoebae. *P. pallidum* WS-320 and *P. violaceum* P-6 were cultivated for six consecutive transfers on autoclaved bacteria, during which time they made approximately 50 generations and showed no decrease in growth rate or any sign of degeneration.

Two experiments were performed to determine whether heat-labile compounds were responsible for the decrease in the growth rate of myxamoebae on autoclaved bacteria: the time of autoclaving the bacteria was varied, and the bacteria were killed by propylene oxide. Figure 13 shows that the heat destruction takes place in two steps. In the first 2.5 to 5.0 min, the decrease in growth was very strong. Further autoclaving caused a marked linear increase in generation time for *P. violaceum* and *D. discoideum*, but only a slight increase for *P. pallidum*. On bacteria killed with propylene oxide, the generation time of the three strains was the same as on bacteria autoclaved for 2.5 min. It seems, therefore, that the first big step in decreased growth is not a specific heat inactivation of some compound, but reflects in some way the transition of the bacterial cells from the living to the dead state.

Hypersonic destruction of the bacterial cell wall resulted in decreased but still fairly good growth of the slime molds, the values being 36% for *P. violaceum* P-6, 52% for *D. discoideum* NC-4, and 54% for *P. pallidum* WS-320, compared with the controls on dead bacteria after a growth period of 44 hr.

Several attempts were made to improve the rate of growth of myxamoebae on autoclaved bacteria. For this purpose, varied concentrations of crude aqueous extracts of living bacteria (broken up by repeated freezing and thawing or by hypersonic treatment followed by Seitz filtration or brief autoclaving) were added to suspensions of dead bacteria. At higher concentrations these extracts were toxic; at lower levels they had no visible effect. No pronounced reaction followed the addition of Eagle's vitamin mixture and amino acids "L" (Eagle, 1955) to a suspension of bacteria autoclaved for 2.5 min. However, when added to bacteria autoclaved for 80 min, on which the myxamoebae of some slime molds grow very slowly, they showed a stimulatory effect in all strains tested (P-6, WS-320, NC-4). The increase in growth was about 100% over a period of approximately 50 hr. Addition of 5% chicken serum, 2% chicken-embryo extract, 2.8% lactalbumin, or Eagle's TC medium "L" (diluted to one-third strength) also failed to show any pronounced effect on the subsequent growth of the myxamoebae. In summary, it was possible in some cases to improve the rate of growth of myxamoebae on bacteria autoclaved for 40 to 80 min, whereas it has not been possible to improve their growth if fed bacteria autoclaved for very short times.

DISCUSSION

The technique developed by Gerisch (1959, 1960) for growing the myxamoebae of *D. discoideum* in liquid culture on living *E. coli* cells has been altered and simplified. Instead of using an air stream to keep the medium in constant motion and to prevent agglutination of the myxamoebae, we attained the same objectives by incubating tube cultures on a rotary shaker. This method was especially useful when only small amounts of nutrient substances were available for tests, and it afforded the additional advantage that many replicates could be run at the same time, thus minimizing variations in results. For certain

experiments and with larger quantities of media, it was convenient to use stirred flasks, especially when the culture vessels were connected to other instruments such as an automatic sampler, a gas supply, etc. Cultures were less aerated, as a rule, using our methods, but this did not seem critical: earlier reports had shown that the myxamoebae grow reasonably well under reduced oxygen tension (Wright and Anderson, 1959; Wescott, 1960), and the generation times obtained in our studies were shorter than those reported by Gerisch (2.9 vs. 3.3 hr for *D. discoideum* V-12). Furthermore, in our experiments additional aeration did not improve growth. Despite the differences in methods employed, our results with *Dictyostelium* and *Polysphondylium* are in good agreement with Gerisch's work.

P. pallidum and *P. violaceum* react very sensitively to the osmotic pressure of the medium. This sensitivity is especially evident in cultures growing on living bacteria, where 0.01 M sodium chloride reduces the growth of *P. pallidum* to a measurable degree. The 8 g/liter of sodium chloride generally incorporated into animal tissue culture media decreases the growth of the same strain on dead bacteria to roughly 10%.

Different strains of cellular slime molds vary markedly in their ability to grow in submerged culture on dead bacteria, but all strains tested thus far do so. The technique is especially suitable for nutritional studies. Compared with the agar-plate technique, it has the triple advantage that sterility can be better controlled, the myxamoebae grow under identical conditions, and their growth can be measured quantitatively.

The increases in generation time of myxamoebae grown on autoclaved bacteria are probably not due to a destruction of a heat-labile substance, since the same effect is observed when bacteria are killed by chemical means. Rather, it seems that the optimal growth of the myxamoebae is somehow related to the active metabolism of the living cell. One possibility, suggested by the experiments, is that the dead bacteria release osmotically active substances in sufficient amount to inhibit markedly the growth of the myxamoebae. Various additions such as bacterial extracts, serum, or embryo extracts did not improve the growth on dead bacteria except when the bacteria had been autoclaved for a very long time.

Of the slime molds investigated, *P. pallidum* grew best on dead bacteria. We shall see in the succeeding paper (Hohl and Raper, 1963) that it also gave the most promising results on culture media devoid of bacteria or any bacterial products.

ACKNOWLEDGMENTS

The writers wish to thank Marianne Hohl for her invaluable technical assistance. The work was supported in part by the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation, and in part by research grants from the National Science Foundation (G-3375) and the National Institutes of Health (C-2119), U.S. Public Health Service.

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