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Microbial Growth Rates in Nature

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INTRODUCTION

The survival of a species in a natural habitat depends ultimately on its ability to grow at a rate sufficient to balance death due to predation, parasitism, or natural causes. Although many aspects of the ecology of a species may be deducible from behavior of the species in culture, it seems evident that at some stage of the study the investigator must return to nature and study the behavior of the population in its natural habitat. This seems even more important now than it was several decades ago because we are now more aware of the tremendous physiological versatility of microbes as evidenced by the existence of biochemical feedback loops, inducible and repressible enzymes, etc. What a species can do in a culture medium is not necessarily what it is doing in its natural habitat. Environmental factors of all kinds may differ drastically in nature from what they are in culture, and they may differ in ways we may not even perceive. This is especially true when we consider that in nature microbes live in microenvironments which may differ widely from the macroenvironment which we are capable of measuring with ordinary instruments and chemical procedures. For instance, nutrient quality and quantity, *p*H, and osmotic pressure are among the factors which can differ between microenvironment and macroenvironment. Hence, it is difficult (if not impossible) to simulate precisely the physicochemical conditions of the natural environment in the laboratory. Furthermore, in nature an organism is always faced with competition from other organisms, difficult to duplicate in the laboratory.

It might seem virtually impossible to measure microbial growth rates in nature, as a result of the small size of microbes and the fact that many species live together. It is the purpose of this review to show that natural microbial growth rates can be measured by a variety of methods adaptable to different kinds of habitats or different kinds of organisms. The review also considers some of the misconceptions about the measurement of growth rates and evaluates some of the pitfalls to successful application of these methods. An attempt is made, when possible, to compare natural growth rates with those of the same species under presumably optimal conditions in the laboratory.

Commitment to Nature

There is a natural tendency for the microbial ecologist to take the easy way out and stay in the laboratory. All sorts of laboratory devices have been constructed which give the illusion of duplicating natural conditions: chemostats,

turbidostats, temperature-gradient blocks, soil perfusion columns. Although these devices permit interesting experiments, they do not replace study of organisms in nature. At some stage, studies in nature must be conducted, preferably before the investigator's ideas about what microorganisms *might* be doing in nature become too firmly fixed. Once some idea of what organisms *are* doing in nature is obtained, ecologically relevant laboratory study of organisms can be conducted in a more meaningful way. Ideally, one should move back and forth from nature to laboratory rather frequently, always checking natural observations with laboratory study and vice versa.

What is Nature?

Any microbial habitat which is not completely controlled can be called a natural habitat. A microbial habitat may be quite tiny. The mammalian intestinal tract, for instance, is not one but many microbial habitats. A single soil crumb may be the home of several kinds of organisms, each in its own niche, and one niche may differ chemically or physically from other niches on the crumb. Except in extreme environments in which selective conditions are so rigorous that populations of single organisms may attain macroscopic dimensions, microbial niches are microscopic ones, and the microscope is an instrument indispensable for this exploration.

POPULATION PROBLEMS

A microbial population is any group of cells of one or more types which can be defined in terms of its extent in space and time. A population may be, for instance, a microbial colony which has a fixed location in space, a species of alga which is distributed widely throughout the plankton of a lake but defined in terms of its characteristic morphology, or the sum total of cells in the rumen, which may be regarded as a single population defined in terms of location.

In terms of growth kinetics, two types of populations can be easily recognized: the steady-state population and the exponentially growing population. The latter, not common in nature, is a closed population of which the members are all growing continuously with no gain from or loss of cells to the environment. The steady-state population may also contain cells which are growing continuously, but cells are being lost from the population at the same rate they are being added. There are, of course, many gradations between exponential and steady state. Thinking about the steady state makes us consider the whole problem of cell migration, which

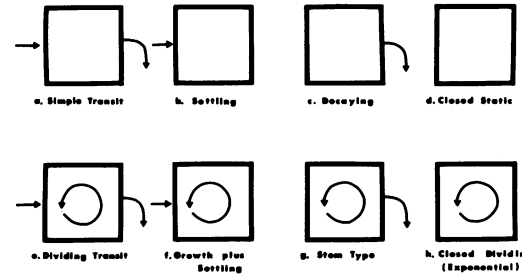

FIG. 1. *Diagrammatic representation of possible cell populations. Arrows outside the boxes indicate cell migration; arrows inside the boxes refer to cell divisions taking place within the system. Based on Cleaver (15).*

can play a significant role in changes in population number. (In fact, one of the most important distinctions between a natural and a laboratory population is that in the former migration can occur.) Examples of different kinds of populations, based on the ideas of Gilbert and Lajtha (22), are shown in Fig. 1.

The upper part of Fig. 1 portrays situations in which no cell division is occurring, and change occurs only due to input and output. An example of a simple transit population (Fig. 1a) is the microflora of the air, or the drift phytoplankton being swept down a river from one lake to another. An example of a settling population (Fig. 1b) is the mud surface of a lake, on which algae may be deposited, or the rectum of a mammal, which is the repository of vast microbial populations from the large intestine. An example of a decaying population (Fig. 1c) is the population in the mammalian intestinal tract after oral antibiotic therapy, in which washout but not growth can occur. Another example of a decaying population is the darkened hot-spring algal mats described by Brock and Brock (11). An example of a closed static population (Fig. 1d) is a dry sample of soil held in the laboratory.

The lower part of Fig. 1 portrays situations in which cell division occurs as well. An example of a dividing transit population (Fig. 1e) is the bacterial flora at a given location in the mammalian intestine, or an algal component able to divide in a moving stream. Of course, virtually any natural dividing population will experience some immigration and emigration, and hence could be assigned formally in this category, although from a practical standpoint it is only when transit is the major component of population change that it becomes relevant to recognize the situation. Depending on the relative rates of immigration and emigration, the dividing transit population (Fig. 1e) grades into the other three

types of growing populations (Fig. 1f, 1g, and 1h). An example of growth plus setting (Fig. 1f) might be the bottom flora of a shallow lake, where an algal species lives both in the water and on the bottom. A *Sphaerotilus* bloom is an example of a stem-type population, to borrow a term from the mammalian cell biologist. In this case (Fig. 1g), immigration is unimportant and emigration by sloughing or release of swarmers is common. The closed dividing population (Fig. 1h) is the familiar exponentially dividing population of the microbial physiologist, and is found only transiently in nature.

It is important to emphasize that a steady-state population as defined by an ecologist is quite different from that defined by a microbial physiologist. The physiologist thinks of the steady state from the viewpoint of the individual cell and defines it as the situation in which "the distribution of each intensive random variable (e.g., cell age or cell protein) does not depend on the time when the sample is chosen" (45), a situation which exists most commonly when the population is in exponential growth. To the ecologist, concerned with the population rather than the individual, exponential growth is anything but a steady state.

ASSESSMENT OF MICROBIAL NUMBERS OR MASS

In any study of microbial growth rates, it is necessary to assess population numbers. The phycologist or protozoologist does this by performing direct microscopic counts of the organisms in a sample from the habitat. If the organisms vary widely in volume, he will not make a simple count, but rather an estimate of the volume of cellular material of a given species. Procedures for such estimates are well established (43, 58). The mycologist may use similar methods (46). Because of the small size of bacteria, the bacteriologist has used direct microscopic counts rather infrequently (16), relying instead on viable counts. Although such methods are useful for physiological and genetic studies in the laboratory on single species with plating efficiencies that approach 100%, they are usually unsuited for ecological investigations. Many studies (e.g., 32, 48) have shown that the viable count is always much lower than the direct microscopic count. Perfil'ev and Gabe (48) give ratios varying from 13,000:1 to 737,000:1. Although low ratios could be due to faulty plating techniques, even under the best conditions at the most about 10% of the bacteria in a natural population may be counted. The reason, of course, that viable counts are done in spite of this serious limitation is that it is often virtually impossible to recognize a bacterial species microscopically, and at least when one has a colony on an agar plate one can subculture it and proceed to an identification. Another reason is that direct microscopic counts are difficult and tedious. Finally, viable counts permit assessment of populations whose densities are too low for measurement by more direct methods.

It should be clearly recognized, however, that viable counts cannot reveal how rapidly organisms are growing in nature. (For an exception to this statement, see the discussion below of Meynell's work.) It is merely necessary to note that a bacterial spore, which contributes nothing to the function of the ecosystem at the time of sampling, will produce a colony on an agar plate. Viable counts are useful in studies of microbial dispersal, and it is in this way that they are best used in ecological studies. The tracing of a source of pollution from a viable count of *Escherichia coli* in a water supply is an example of this kind of study.

If we cannot assess bacterial numbers in a natural habitat by viable counts, how then are we to do it? First, direct microscopic counts are often possible when the bacterium in question is large or morphologically distinct. Examples of this approach are the enumeration of *Chromatium* in lakes (35) or of *Leucothrix mucor* attached to seaweeds (10). Second, identification may be possible at the microscopic level by the use of fluorescent-antibody procedures (26, 52). Third, we may be dealing with habitats in which only one or a restricted number of kinds of organisms are present, so that species identification is no problem (2). Admittedly there are cases in which none of these situations obtains, but new methods can probably be devised.

El-Shazly and Hungate (18) obtained a measure of the microbial mass in a rumen fermentation by measuring the rate of gas production of samples of rumen contents containing a rate-saturating quantity of fermentable substrate, so that gas production was a function only of population size. The absolute population size could not be measured, but *relative* changes in population density could be. A precisely analogous method can be used by those working with photosynthetic organisms. The rate of photosynthesis (measured with $^{14}CO_2$) can be measured under conditions of saturating light intensity. Changes in population density will then be reflected by changes in the rate of $^{14}CO_2$ uptake.

A cell constituent which may be of considerable value in determining relative population densities of actively metabolizing microbes is adenosine triphosphate (ATP) (29). Sensitive techniques

are available for its measurement, and ATP concentration should be proportional to biomass. Of course, only in natural systems where microbes constitute the majority of the biomass could ATP determinations be used to estimate the relative population density of microbes.

Chlorophyll has been used frequently as an index of algal biomass, but is not a reliable one because chlorophyll content per cell may vary widely as a function of light intensity (12). Epply (19) described a method for measuring standing crop by extrapolating the photosynthesis-rate curve back to zero time.

Estimating population size of filamentous organisms is more complex, but at least the temptation to use viable counting procedures does not arise. The direct way to measure lengths of filaments is to enlarge the field of view either by photography, projection, or drawing with camera lucida, and then to measure the lengths of filaments with a ruler or map reader. This approach has been used by soil mycologists (46). A simpler way, and one which is probably just as accurate, is to use a stereological technique. A grid is thrown across the field and the number of intersections of filaments with lines of the grid is counted. Olson (43) described five different stereological procedures, each suitable to a particular system or providing a particular degree of accuracy. We have used one of his methods for assessing aggregate lengths of filamentous bacteria on artificial substrates with considerable success (3), and we can recommend it for convenience, rapidity, and relative accuracy.

DEFINING BOUNDARIES OF THE HABITAT

Before a study is begun, the boundaries of the habitat must be defined. Initially one must decide on the scale of the investigation. For study of microbial growth in soil, for instance, a dimension as large as a field or as small as a soil crumb could be selected. For a lake, the whole body of water could be investigated, or any fraction of this body, such as the planktonic regions, the littoral, the thermocline, or the bottom sediments. Even in a habitat as sharply bounded as the rumen, it might in some cases be useful to consider subsections (e.g., rumen wall, food particles) in which particular species or processes might be concentrated.

For study of the growth rate of a single species, it is usually preferable to examine the smallest habitat encompassing the boundaries or the field of influence of the organism. Thus *L. mucor* lives as an epiphyte of benthic algae on the littoral of sea coasts (6). It would be unprofitable to study the whole vast littoral region rather than the specific seaweeds with which *L. mucor* is associated.

It is essential to know the type of distribution which the organism has throughout the habitat. Animal and plant ecologists (27, 38) use the terms "dispersion" or "pattern" when referring to this aspect. If a population is randomly dispersed, its distribution will follow a Poisson distribution. Random dispersion is rare in natural populations, the species more frequently being patchy or clumped. In such cases, the variance and the mean are not equal, as in the Poisson, and departures from randomness can be detected by deviations of the distribution from a Poisson series. Greig-Smith (27) gives a variety of statistical tests which can be used to detect such differences and to express them quantitatively. In general, all these methods use data derived by counting the number of individuals in a series of quadrats.

A quadrat is a real or imaginary area of known size which is thrown across the field. Despite its name, a quadrat need not be square but can have any convenient shape; for example, the area encompassed by a microscope field is a quadrat. It is obvious that the quadrat size must be large enough so that distributions obtained are not biased by quadrat size. This point, discussed in detail by Kershaw (34), is illustrated in Fig. 2. It can be seen that if the quadrat size is either too small or too large, patchiness will be missed. The ideal quadrat size for detecting patchiness would have an area approximately equal to the area of the clump, since some quadrats would contain large numbers of organisms and others would be virtually devoid of organisms.

To assess growth rates over the whole extent of the habitat, it is important to use quadrat sizes large enough to eliminate changes in number caused by patchiness. At the same time, very large quadrat sizes make the work of counting unnecessarily tedious.

It is essential to study habitats in which the organism of interest is actually growing. Many organisms are carried by streams or currents far from the site where they reproduce. For instance, *Staphylococcus aureus* grows in the kidney, forming so-called foci of infection. Cells from these foci are sloughed into the blood and carried to distant parts of the body. It is easy to assess staphylococcal numbers in the blood stream, but it would be incorrect to use changes in these numbers as a measure of staphylococcal growth rates in the kidney. In algal blooms, the cells often float to the surface and are carried by wind and current to quiet backwaters where they accumulate in vast numbers. The factors that con-

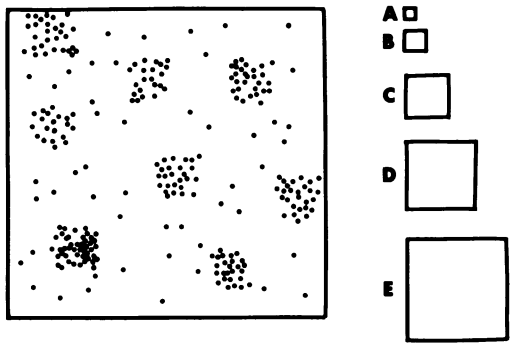

FIG. 2. *Relationship between quadrat size and the detection of nonrandom distribution. If the population were sampled with quadrats A or B, only slight nonrandomness would be seen, with C very marked nonrandomness; with D and E the distribution would appear progressively more random. Thus, the most marked demonstration of nonrandomness would be with a quadrat area about equal to the area of the clump. Based on Kershaw (34).*

trol algal blooms can not be ascertained by studying algae in such backwaters rather than in the part of the water where the algae are actually growing.

ARTIFICIAL SUBSTRATES

The use of artificial substrates greatly simplifies many studies in microbial ecology. The substrate which has been most commonly used in both aquatic and soil microbiology is the glass microscope slide (14, 53). The main advantage is ease of microscopic examination, but an additional advantage for rate studies is that one knows precisely the time microbial growth *could* commence. For studies on colonization and succession artificial substrates are ideal. The substrate may be both physically and chemically analogous to many natural substrates such as rocks and minerals. A wide variety of materials (e.g., plastics) other than glass can be used (53). If necessary, even opaque materials can be used in conjunction with incident fluorescence microscopy. In fouling studies, the artificial substrate is at the same time the habitat of interest.

In soil or other heterogeneous systems, microscope slides are difficult to use because (i) they cannot be removed and replaced without disturbing the environment; (ii) the opacity of the particulate material makes microscopy difficult; and (iii) it is difficult to make microscopic observations of living organisms, so that subsequent isolation of an organism of interest is usually not possible. Because of these and other difficulties, Perfil'ev and Gabe (48) devised special flat-glass microcapillaries which could be inserted into the habitat for colonization and then removed and examined periodically (Fig. 3). These flat capillaries, called by them "pedoscopes" (if used in soil) or "peloscopes" (if used in mud), are far superior to round capillaries for microscopy, and have been used in the Soviet Union for many years. Their use will no doubt become more general as a result of the appearance of English translations of Perfil'ev's two books (47, 48). Through the use of these capillaries, Russian workers have discovered and studied new bacteria that live at the mud-water interface: *Lieskeella*, *Dictyobacter*, *Cyclobacter*, *Trigonobacter*, *Metallogenium*, and others. (The taxonomic validity of all of these entities is not accepted by all bacterial taxonomists.) In addition, new bacterial genera have been described from soil and water. Further, with the use of microcapillaries, Perfil'ev has been able to observe the microzonation which develops at the mud-water interface. For instance, in the mud of Lake Khepo-Yarvi, eight microzones developed over a vertical distance of 2 mm. From the surface down, these were photosynthetic zone (diatoms), iron oxidation zone (*Ochrobium* and *Gallionella*), predatory bacterial zone (*Dictyobacter*), *Azotobacter* zone, filamentous bacterial zone, *Cyclobacter* zone, *Lieskeella* zone, and sulfide oxidation zone (*Thiospira*). Below the eighth zone, conditions became highly reducing, as shown by the presence of FeS.

The value of flat-glass capillaries for many ecological studies is clear. The book by Perfil'ev and Gabe (48) should be consulted for details of methods and results, as well as for techniques used to actually construct the capillaries. In addition, this book has a detailed discussion of how capillaries can be used in continuous-flow systems and with micromanipulators.

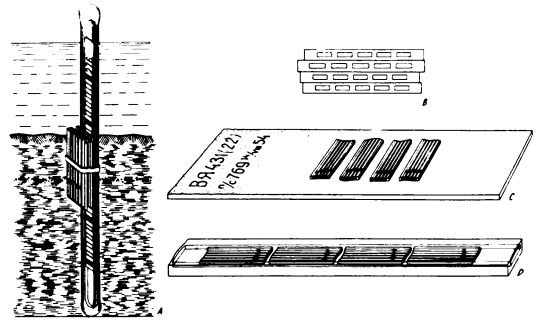

FIG. 3. *Capillary peloscope of Perfil'ev and Gabe (48). A. Capillary peloscope inserted at the mud-water interface. B. Cross section of typical capillaries. C. Capillaries mounted on a microscope slide for observation. D. Capillary cells in a protective channel with fixing fluid.*

USE OF THE MICROSCOPE IN NATURE

Two approaches are possible. One can take the microscope to the field—in the sense of actually submerging it in the environment—or one can bring the field to the microscope. The first approach is fraught with difficulties and has been used only rarely. Staley (54) immersed a phase microscope in a small pond in Michigan and used it to study algal growth. This technical tour de force may serve as an example of the great difficulties with which the microbial ecologist is faced. In the first place, even with a submerged microscope a glass slide was necessary and served as an artificial surface. The slide was immersed in the water near the microscope and was transferred periodically to the microscope stage for observation and photography. To permit periodic viewing without diving, the microscope was placed at the surface of the water, with the eyepieces emerging from the pond. Staley watched and periodically photographed algae and bacteria which appeared on the slide, and thus determined the manner and rate of growth. Although in principle the same or a modified technique could be used in more rigorous environments such as deep waters, rivers, the ocean, perhaps even hot springs, in practice the difficulties arising are probably too great in terms of the information gained. A much simpler approach is to bring the submerged glass slide from the field to the microscope and to observe and photograph it by using a water-immersion lens. This was the approach used by Bott and Brock (3) to study bacterial growth rates in a small greenhouse pond. Microscope slides which were marked with a diamond pencil at a number of locations were submerged in the water. At intervals, the slides were brought to the surface and carried submerged to the microscope in glass petri dishes. Locations containing organisms were photographed and the slides were then returned to the pond. The temperature was not altered significantly and the slides were out of the pond for just a few minutes. Since at least 5 mm of water was present over the surface of the slide, presumably the organisms, 0.5 to 1.0 μm in thickness, did not become disturbed. An alternate method would be to use the microcapillaries of Perfil'ev and Gabe (48), discussed earlier.

In soil, experiments of this type are considerably more difficult due to the nature of the substrate. A single slide cannot be removed and reinserted periodically without disturbance. Microscopy of undisturbed soil particles is even more difficult. Recently, Casida (13) made a useful advance towards direct microscopy of undisturbed soil particles. The procedure involves the use of incident illumination of the soil particle by light which passes through the objective in reversed fashion. The bacterial cell so illuminated diffracts the light, and light of some wavelengths returns through the objective to the ocular. Various colors are seen, the cell thus being differentiated from the dark background of the soil. However, actively growing bacteria do not exhibit this diffraction phenomenon, so that the technique permits observation in soil only of those cells which are in a dormant state. Microorganisms larger than bacteria cannot easily be seen either. Casida concludes that the technique will permit continuous observation of the growth, activities, and interactions of soil microorganisms, although if only dormant cells are observed it is not clear how one can study growth in situ; no growth rates have as yet been presented. If nothing else, the report of this technique may perhaps encourage further study and experimentation with the light microscope, the most powerful tool of the microbial ecologist.

MEASUREMENT OF GROWTH RATE OF SINGLE ORGANISMS

The most direct method of measuring growth rate is to measure the size or mass of a single organism at various times. This method, which is relatively easy to use in plant and animal ecology, is difficult to use in microbial ecology for several reasons. (i) It is often difficult or impossible to return periodically to precisely the same organism. (ii) Only nondestructive measurements can be used, such as measurement of size. Mass, the most important ecological parameter, may not relate too precisely to size. Interference microscopy does permit an estimate of cell mass, but is difficult to apply routinely or to very small cells. (iii) The procedure is tedious, especially with slow-growing organisms, as one must wait for long periods of time to obtain data on even a single cell. During the wait, the microscope may be tied up and cannot be used for other things. (iv) Because only a single organism is studied at a time, the data obtained may not be representative of the population as a whole.

There has been a considerable amount of work involving measurements of growth rates of single cells (usually bacteria or yeasts) in pure culture. This work, most recently reviewed by Painter and Marr (45), provides some clues concerning the growth of microbes in nature. Painter and Marr discuss some of the difficulties of accurately measuring growth rates of individual cells microscopically from changes in cell size. For cells the size of bacteria, the error of measurement

is such that distinction between linear and exponential growth cannot readily be made. The consensus seems to be that the individual bacterial cell may grow exponentially, whereas the individual eucaryotic cell does not. For most ecological work, the distinction may not be too important. What is needed is merely a measure of how long it takes for a young cell to increase in size and to divide.

In his studies with a submerged microscope, Staley (54) observed the growth of *Chlorella* in a small Michigan pond. During the day a single cell increased in size, and at night this enlarged cell divided into four daughter cells. This is identical with the behavior of *Chlorella* cultures growing in alternating light and dark in the laboratory (56). Not all of the cells observed by Staley managed to complete division in 24 hr. In certain cases, if a cell did not complete division during the first night it continued to enlarge during the second day and then completed division the following night.

Measurement of growth rates of filaments involves the same principle as that of unicells, but microscopic recognition of the growing element is easier. No study of *direct* measurement of filament growth in nature has come to my attention, although much work on growth of single fungus filaments in laboratory culture has been done (50).

GROWTH RATES OF DEVELOPING MICROCOLONIES

The growth rate of microorganisms that form microcolonies in nature can be estimated by counting the number of cells per microcolony at different time intervals. Counting numbers is easier than measuring the sizes of single cells; hence, it is more convenient for routine work. When evaluating counts of microcolonies, one should keep in mind that immigration or emigration of cells could have occurred during the intervals between counts.

Bott and Brock (3) determined growth rates of aquatic bacteria developing at marked locations on microscope slides immersed in a small pond. The changes observed during the growth of a single microcolony are shown in Fig. 4. Considerable heterogeneity in developing microcolonies was observed. Some colonies never got beyond the two- to four-cell stage. Others developed into larger aggregates, after which most of the cells in the colony lysed. In others, the cells spread out after the colonies reached the 16- to 32-cell stage, making identification of the colony difficult. Only a few colonies developed to really large size (e.g., 128 cells or larger). Presumably, many of the organisms which initiated colonies on the

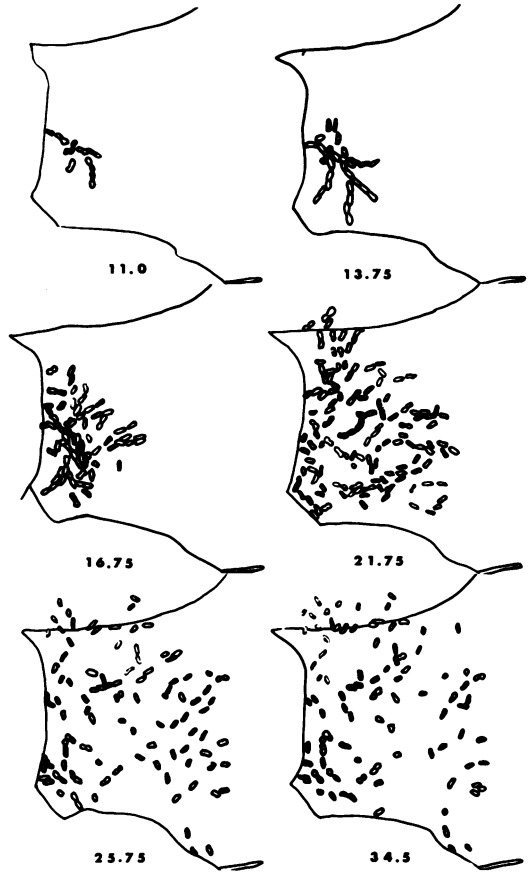

FIG. 4. *Development of a single microcolony on a glass slide immersed in a small pool. Time is expressed in hours after immersion. Drawings from photomicrographs of Bott and Brock (3).*

slide were not well adapted to a sessile existence, and they succumbed after utilizing organic material adsorbed to the slide in their immediate environment. However, even the organisms which were well adapted (as shown by their formation of larger colonies) never completely dominated the habitat because grazing organisms such as protozoa eventually moved in and consumed them. Doubling times obtained by counting on the photographs the numbers of cells per microcolony were in the range of 2 to 10 hr.

In this study (3), both immigration and emigration were observed. Not infrequently, a cell would undergo division and one of the daughter cells would swim away; the remaining cell would remain attached to the glass and undergo further division. This phenomenon is common in stalked bacteria such as *Caulobacter* (49), but was also seen for bacteria without obvious stalks. The technique of Helmstetter (28) for obtaining

growth-synchronized populations of bacteria depends on the fact that the parents remain adherent to a support and the just-formed daughters are washed free.

However, in flowing-water habitats the organisms are usually types in which both products of division adhere tightly to the substrate, so that emigration is of little consequence; the evolutionary advantage of adhering tightly to the substrate in this situation is obvious.

In estimating growth rates, immigration is probably a lesser source of error than emigration; if microcolonies are widely spaced, the probability that an immigrant will settle within a colony is fairly low. Two biological factors which could affect immigration are antibiosis, which could reduce it, and chemotaxis, which could increase it. A technique involving ultraviolet radiation for quantifying immigration rates is described below.

For routine work, it would be desirable to be able to quantify growth rates of microcolonies without the complications involved in removing the slides, locating and photographing the colonies, and replacing the slides, especially when all of these operations must be done quickly and without disturbance to the organisms. To obviate these problems, Bott and Brock (3) immersed a series of slides at a single location and removed two or three at different time periods. On each slide, the cell count in a large number of microcolonies was made, and the average number of cells per microcolony was calculated for that time period. By plotting numbers of cells per microcolony at different immersion times, the growth rate could be calculated. Assuming that the organisms in the developing microcolonies are sufficiently distinct so that the microcolonies arising from cells belonging to the same species can be recognized microscopically, the lumping of data from different microcolonies is valid as a measure of the growth rate of a single organism. If, however, such recognition is not possible, the growth rates obtained represent the average growth rate for the aggregate of microbial species on the slide. For some ecological work, this latter information is still useful.

In many aquatic habitats, bacteria do not develop on the slides as discrete microcolonies but are scattered more or less randomly. This distribution might arise through growth if daughter cells moved away from mothers before settling down. On the other hand, it could arise as a result of immigration in the absence of growth. To distinguish growth from immigration, Bott and Brock (2, 3) employed ultraviolet radiation. Slides which were immersed were irradiated with a germicidal ultraviolet source at regular intervals during the experiment, the intervals calculated to be about equal to the generation time of the population. Thus, newly attached immigrants were killed before they divided. The rate of increase of cell numbers on unirradiated slides is a function of both immigration and growth, whereas on the irradiated slides it is a function only of immigration.

Several conclusions could be drawn from these irradiation experiments. Microcolonies never developed on irradiated slides, showing that microcolonies arose as a result of growth. In most habitats, immigration is quantitatively unimportant. The rate of increase in cell number, even if distribution on the slides is essentially random, is caused primarily by growth on the slides. Therefore, if the immigration rate is not subtracted no serious error will be introduced in calculating growth rates. It should be noted that the habitats studied were relatively unpolluted and microbial drift through the water was low. In habitats in which drift is high, immigration may be more significant.

Modifications of the ultraviolet radiation technique can be envisaged which would make it adaptable to other habitats. In soil, which ultraviolet radiation would not penetrate, gamma radiation might be substituted. In some other habitats, germicidal chemicals might be used. The only requirement is that the agent in question not cause cells to be liberated from the surface, since if that occurred the immigration rate would be underestimated. Bott and Brock (*unpublished data*) tested a number of chemicals, including ethyl alcohol, formaldehyde, mercuric bichloride, and hydrochloric acid, but these proved unsatisfactory either because they modified the surface in such a way that attachment was inhibited or they caused detachment.

GROWTH RATES OF FILAMENTOUS ORGANISMS

Various methods (43) are available for quantifying filamentous organisms and can be used in conjunction with slide immersion and ultraviolet radiation to measure growth rate. Representative data on the growth rate of *Sphaerotilus* on glass slides (4) are given in Fig. 5. The glass slides were colonized initially by unicellular swarmer cells. In the ultraviolet-irradiated slides no *Sphaerotilus* filaments appeared, whereas on the unirradiated slides colonization by swarmers was followed by outgrowth of *Sphaerotilus* filaments. Growth rates for filamentous bacteria living in hot springs have also been measured in this way (Bott and Brock, *unpublished data*). In most of these habitats, both unicellular and filamentous bacteria developed on the same slides, and the doubling times of both kinds of bacteria were fairly similar.

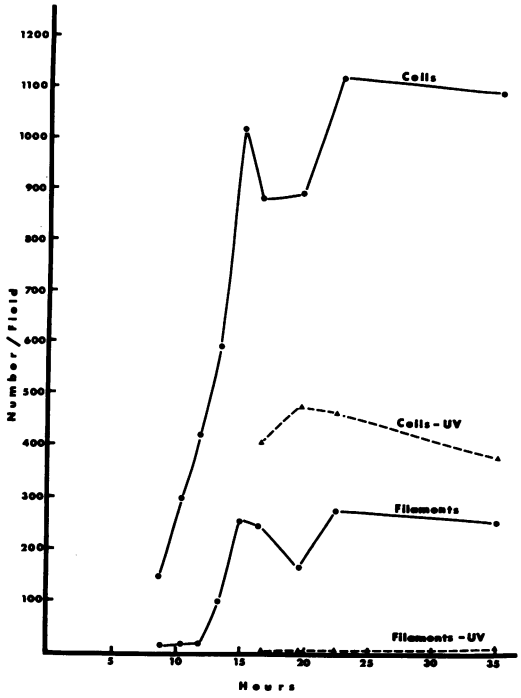

FIG. 5. *Quantitative counts of total Sphaerotilus cells and of filaments on glass slides immersed in a small stream. On the ultraviolet-irradiated slides, single cells never develop into filaments. From Bott and Brock (4).*

When expressing the doubling time of the filaments, the value given is the rate of increase in the aggregate length of filaments per unit area. This could be due either to increase in lengths of existing filaments or to the increase in number of attaching filaments. Again, studies using ultraviolet radiation permit these alternatives to be distinguished. In all cases studied, growth was due primarily to increase in length of filaments. On irradiated slides only short filaments were observed. On unirradiated slides, the lengths of filaments got progressively longer as immersion time increased. The rate of increase of length of filaments was exponential over two decades, suggesting that growth was occurring along the whole lengths of the filaments, rather than only at the tips as it does in filamentous fungi. If the latter growth habit occurred, rate of increase should have been as the square rather than the exponential.

METHODS BASED ON ANALYSIS OF THE CELL-DIVISION CYCLE

The typical division cycle of a eucaryotic cell is given in Fig. 6. In this cycle, mitosis can be recognized morphologically and deoxyribonucleic acid (DNA) synthesis can be recognized autoradiographically by the use of radioactively labeled thymidine. In many cell types, the time of mitosis represents a constant fraction of the total cell-division cycle. If this is true and if the time occupied by mitosis is known, the division rate of a population can be calculated by a formula derived originally by Crick (*cited in* 30), time of mitosis/total time of cell-division cycle $\times$ $\log_e 2 = \log_e (1 + 2R/1 + R)$, where R is the fraction of cells in mitosis. If interphase is relatively long and R is thus small, this formula reduces to time of mitosis/total time of cell-division cycle = $1.44R$. The use of this formula assumes that the rate of entry of cells into mitosis is uniform.

This approach was used by Warner (57) to estimate division rate of the protozoan *Entodinium* in the rumen. The time for mitosis was determined by microscopic observation of entodinia using a warm stage; a value of about 15 min was obtained. It was assumed that this value holds for all cells of that species in the population. The fraction of cells in mitosis (r) was then counted at different times and the time for cell division was thus obtained. The minimum mean doubling time found was 5.5 hr, which corresponds to a maximum division rate of about four generations per day. However, dividing forms were much more common at night than during the day. The true division time of the population can be calculated by averaging the various division times over the 24-hr period; it is 15 hr (*see* 31). The maximal growth rate obtained for entodinia in culture was one division every 2 to 4 days, suggesting that conditions in culture are far from optimal for multiplication.

Labeled Thymidine Methods

The duration of the cell cycle can also be estimated by labeling with radioactive (^3H or ^{14}C)

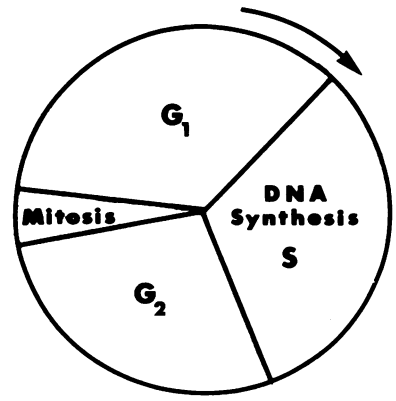

FIG. 6. *Cell cycle in an eucaryote. Arrow indicates the direction in which cells progress in the cycle.*

thymidine. The problem of determining growth rate of mammalian cell population in vivo by thymidine labeling is, in principle, the same as the problem of determining growth rate of a microbial population in nature, and the book by Cleaver (15) provides a detailed summary of the concepts involved.

If a population is labeled with a brief pulse of tritiated thymidine, only those cells in S phase (i.e., engaged in DNA synthesis) will be labeled. After continued growth of the population, the labeled cells will pass through mitosis as a wave, and this fraction can be estimated by preparing autoradiograms at different times and counting the fraction of labeled cells which are in mitosis. At least in mammalian cells, mitotic figures can be easily recognized. The results to be expected from the ideal case and the results obtained in an actual example are given in Fig. 7. Initially, there are no labeled mitoses until a time equal to G_2 has passed, after which the fraction of labeled mitoses rises to 1.0 in a period equal to the duration of mitosis (M). The labeled fraction remains at 1.0 for a period equal to the duration of the S phase minus the duration of mitosis (S − M), and subsequently falls to zero. The second wave of labeled mitoses appears after a further time equal to $G_1 + G_2$. It is thus possible to calculate the duration of the various phases from the time periods exhibited by the graph. Although the shape of the experimental curve does not precisely follow the ideal, it is close enough for most practical purposes.

The limitations of any technique involving the use of radioactive thymidine should be kept in mind. It must be known that the population in question assimilates this compound and incorporates it only into DNA. If there is a large pool of nonradioactive thymidine, this will affect the kinetics of labeling; consequently, it is essential at the end of the pulse to add an excess of nonradioactive thymidine, which dilutes any labeled material remaining in the pool. It is essential to know that the tritiated thymidine itself does not alter the cell cycle (e.g., through radiation damage). The labeling time must be kept short, but it must be long enough to permit labeling sufficient for detection on the autoradiograms. The technique can be applied only to systems in which the number of dividing cells is sufficiently high that the fraction of labeled mitoses can be determined accurately.

If pulse labeling, which often requires manipulation of the culture, is not possible, continuous labeling can be used. With this method, the isotope is added once and maintained in excess throughout the labeling period. To determine the

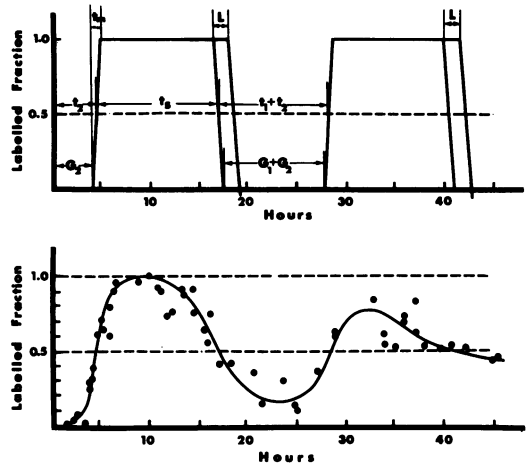

FIG. 7. *Fraction of labeled mitoses as a function of time after a pulse of ^{3}H-thymidine. Upper: ideal case with no intercell variation. Abbreviations: L, duration of labeling; t_m, time for mitosis; t_s, duration of S phase; t_1, duration of G, plus one-half of T_m; t_2 duration of G_2 plus one-half of t_m; G_1 and G_2 as on Fig. 6. Bottom: actual data for mouse L cells. G_1, 5.7 hr; S, 12.2 hr; G_2, 4.4 hr; M, 0.9 hr; T (total cell cycle time), 23.2 hr. Taken from Fig. 4.5 of Cleaver (15).*

parameters of the cell cycle with this method, three functions must be determined: the fraction of labeled mitoses, the fraction of labeled cells, and the average grain count per cell. A typical continuous-labeling experiment is shown in Fig. 8. The upper curve, showing the rate of increase in labeled mitoses, is used to calculate G_2, as in the pulse-labeling technique. The fraction of labeled cells increases linearly (middle curve) as a result of the entry of cells into the S period. With populations in exponential growth, there are more young cells than old; hence the rate of entry of cells into S is greater than the rate of cell division. For this reason, the rate of increase in labeled cells is faster than the rate of cell division and cannot be used directly as a measure of division rate. The labeled fraction will reach 1.0 at a time equal to $G_1 + G_2$, and since G_2 was estimated already G_1 can now be calculated by using the data obtained in the middle curve. The maximum amount of radioactive thymidine which a cell can incorporate in one cycle (estimated by the average grain count) corresponds to a single complete round of DNA synthesis. Since this takes the whole S phase to complete, the time it takes for the average grain count per cell to reach a constant level corresponds approximately to the S period (lower part of Fig. 8). [In actual fact, this procedure slightly overestimates the duration of the S period; the complications are discussed by Cleaver (15).]

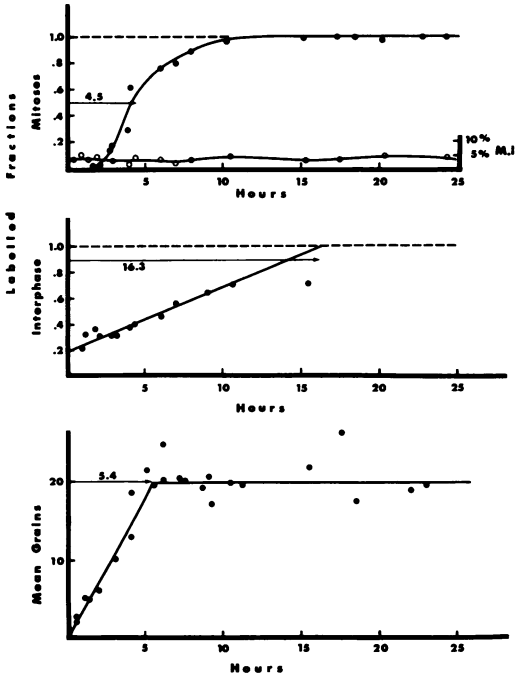

FIG. 8. *Continuous-labeling experiment with ^3H-thymidine and human skin epithelium cells. The upper curve is used to determine t_2 (4.5 hr) in the same manner as in Fig. 7. The middle curve is used to estimate $t_1 + t_2$ (16.3 hr); thus both t_1 and t_2 can be calculated. S (5.4 hr) is estimated from the lower curve. The other parameters can then be calculated. Taken from Figure 4.6 of Cleaver (15).*

An alternative method of measuring the rate of entry of cells into the S phase involves a double labeling technique, a pulse of tritiated thymidine being used for the first labeling followed after a defined time interval by a pulse of ^{14}C-thymidine. Autoradiograms can be prepared by using a double-emulsion technique which permits counting separately radioactivity due to tritium or carbon-14. The interval between exposures to the two labels should be less than $G_2 + M$ to ensure that labeled cells do not divide during the period of labeling (36).

If it is possible to determine the DNA content per cell by microspectrophotometry of Feulgen-stained autoradiograms, the relative duration of the four phases of the cell cycle can be determined more directly, since cells in G_2 will have twice the DNA content of cells in G_1. The population need be labeled only for a brief period before fixation so as to label the S-phase cells. By microspectrophotometry, the unlabeled cells can be divided into G_1 and G_2; the mitotic cells are detected cytologically, and the S phase cells by autoradiography. In this way, the fraction of the cells in each of the four phases is estimated and the *relative* durations of the four phases can be determined (*see* p. 123 of reference 15 for details). To convert these relative values into absolute ones, it is necessary to determine by other methods the duration of one of the stages of the cell cycle in absolute terms. The advantage of this method is that it requires only brief labeling and only a single sample of the population. The disadvantage is that it requires microspectrophotometry, which is laborious and can be performed only on fairly large cells.

Although the methods described above have been applied extensively to mammalian systems, they have been used only rarely with eucaryotic microorganisms, usually with pure cultures [*see* Cleaver (15) for references]. However, the potential value of these methods for microbial ecology is clearly so great that their description here appears justified.

GROWTH RATES OF PROCARYOTES AS MEASURED BY THYMIDINE AUTORADIOGRAPHY

The measurement of the growth rates of procaryotes presents some new complications because procaryotes do not exhibit the typical mitotic cell-division cycle described above for eucaryotes. In the bacteria which have been studied, DNA synthesis in exponential populations occurs throughout virtually the whole of the cell cycle, being interrupted only briefly at the time of cell division (37). Consequently, if a pulse of tritiated thymidine is given to a growing bacterial population, virtually all cells become labeled to some extent, in contrast to the situation in eucaryotes in which only cells in S phase get labeled at all, and these heavily. A second problem is that since procaryotes do not divide by mitosis, it is impossible to evaluate microscopically the fraction of cells in division. Last, because of the small size of most procaryotes and the low DNA content per cell, grain counting on autoradiograms is difficult; even heavily labeled cells have only a few silver grains over them.

If grain counting is possible, growth rate can be estimated by a continuous-labeling method. Referring to Fig. 8, it can be seen that the time required for the mean grain count per cell to reach saturation is approximately equal to the length of the S phase, and if the S phase occupies 90 to 97% of the cell cycle (the situation in *E. coli*), the length of the cell cycle (virtually the same as the doubling time) can be estimated.

It would be desirable to have methods which did not require grain counting. An approach I

used (10) was to measure the rate of accumulation of labeled cells in the population during continuous labeling. The marine bacterium *L. mucor* was used in this study. This is a large, filamentous epiphyte of seaweeds which is readily recognized in natural material. Since the filaments project perpendicularly from the surface of algal fronds, microscopy and preparation of autoradiograms is relatively easy. In growing laboratory cultures, the rate of accumulation of radioactive cells is linear with time at least until 80% of the cells are radioactive. (Some cells never become radioactive even after long time periods, so that if incubation with tritiated thymidine is continued indefinitely the rate of accumulation of radioactive cells falls off.) The rate of accumulation of radioactive cells is proportional to the doubling time of the culture, and about 1% of the cells get labeled in 0.002 generation. This relationship, determined for a pure culture, could then be used to estimate doubling time in nature (10). With this technique, one assumes that the rate of accumulation of radioactive cells is linear and begins without a lag, just as it does in pure cultures, and that nonradioactive thymidine, which might dilute the radioactive material, is absent from seawater. It is desirable to analyze samples incubated at several different times to ensure that the rate of incorporation is indeed linear in nature.

This technique requires a study with laboratory cultures growing at known rates so that the field data can be converted into real doubling times, and it requires the assumption that there is nothing intrinsically different about the way cultures and natural samples incorporate tritiated thymidine.

For determinations of relative rather than absolute growth rates, preliminary studies with laboratory cultures are not necessary. From the labeling rates of different natural populations it is possible to deduce which populations are growing fastest. For some studies, such relative rates suffice. However, to determine the contribution of a particular organism to food chains, absolute rates are essential.

Ecological Approximations for Labeling Experiments

The labeling of DNA is a particularly valuable technique for the study of growth rates because DNA is the only macromolecule in the cell which does not turn over and because synthesis does not occur in nongrowing cells. In exponentially growing populations, in which turnover is negligible, the rate of synthesis of any macromolecule could be measured because all materials are synthesized at the same rate. Since even in steady-state populations turnover and resting synthesis may be low, the measurement of the rate of synthesis of some macromolecule other than DNA might be acceptable as a first approximation for ecological work.

A point which needs to be emphasized is that in any labeling experiment the time of incubation with the isotope must be kept short (i) to prevent any secondary changes from taking place during the time the population is confined to the bottle, (ii) so that the concentration of isotope always remains in excess, and (iii) so that the experiment is terminated before any recycling of the label has occurred.

Rate of Appearance of Unlabeled Cells

Another radioautographic method which has some advantages over those described above involves measuring the rate at which unlabeled cells appear in a population which is fully labeled and then placed in medium which lacks label. Because of the semiconservative replication of DNA, after one cell division the specific radioactivity of each of the two offspring will be halved; consequently, one can measure growth rate by measuring the time taken for the grain count to decrease by half. The advantage of this method is that the labeled population can be placed under completely natural conditions, eliminating effects of confinement to bottles or to effects of the isotope itself. The disadvantages are that the population must be removed from its natural environment for labeling and that after several cell divisions the mean grain count will be reduced to such low levels that an accurate count will not be possible. Thus, the method seems best adapted for studying the behavior of species after introduction into foreign environments. This method apparently has not been used in ecology, although it is widely used by mammalian cell biologists (15). The genetic method of Meynell (discussed below) is formally equivalent to this isotope-dilution method.

In bacteria or other small organisms in which grain counting is difficult, a modification of this method might be to measure the rate of appearance of unlabeled cells in a fully labeled population. After the first cell division, all cells will still be labeled, but the labeled fraction will decrease by half after the second and subsequent cell divisions (Fig. 9). By counting the labeled and unlabeled cells after various periods of time, the doubling time of the population can be calculated.

Although for both of these methods a fully labeled population is desirable, the methods can be less accurately applied to partially labeled populations. This would be of considerable value

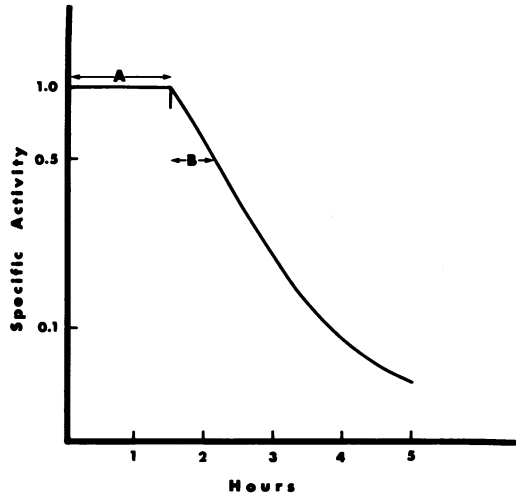

FIG. 9. *Change in specific radioactivity of a population as a function of time after labeling. "A" is the time that elapses before labeled cells pass through the nondividing compartments and begin to decrease in radioactivity. "B" is the time for the specific radioactivity to decrease by one-half, which is equivalent to the doubling time. After Fig. 7.5 of Cleaver (15).*

if full labeling required excessively high isotope concentrations or long incubation times.

GENETIC METHODS

An interesting method using genetically marked cells was devised by Meynell (40, 41) to measure the growth rates of bacteria in the animal body. As Meynell notes, bacterial growth in vivo is usually much slower than it is in laboratory cultures. This could be either because the animal is an unfavorable habitat for bacterial growth or because bacteria are being killed in vivo so that the division rate is greater than the rate of increase of the viable count. These two possibilities can be distinguished only if a method is available to measure bacterial growth when some cells are dying. The technique which Meynell used was to introduce a nonreplicating genetic marker into the bacteria before inoculation into the animal. As shown in Fig. 10, at each cell division the fraction of the population which contains the label will be halved. One determines the total population and the population with the genetic label at different time periods; from the rate at which the marker is lost, the doubling time can then be calculated. This method is formally analogous to the isotope dilution method described above, but it is simpler because it eliminates autoradiography and direct microscopy, the labeled and unlabeled populations being assessed by plating procedures.

In his first work, Meynell (40) made use of the fact that when a lysogenic bacterium is superinfected with an appropriate mutant of its prophage, the mutant enters the bacterium but does not replicate. Its presence can be assessed after induction by plating on an indicator bacterium which is specific for the superinfecting phage. Preliminary experiments showed that the marked population did behave as predicted during cell division up until the 10th generation, after which the proportion of marked bacteria decreased at a rate less than predicted, probably because about 0.1% of the original bacteria were lysogenized by the superinfecting phage so that replication could occur. Mice were inoculated intravenously with *E. coli* K-12 lysogenized with phage lambda *b* and superinfected with lambda *hc*. Within 30 min, the bacteria had cleared from the blood and were lodged in the liver and spleen. Assessment of total viable counts showed that the viable count dropped about three decades in the first 8 hr and did not decrease farther over the next 48 hr. The proportion of labeled organisms remained constant over the duration of the experiment, showing that no replication occurred.

In a second study (42), Meynell applied this technique to a study of the replication, killing, and excretion in the feces of *Salmonella typhimurium* which had been inoculated into the mouse orally. The genetic label was a histidine gene introduced in the abortively transduced state. Since the gene in an abortive transductant does not replicate, it is passed to only one of the daughter offspring at division. The proportion of cells in the population containing the gene is determined by plating on medium without histidine and scoring for the tiny colonies formed by the abortive transductants. Since excretion was a

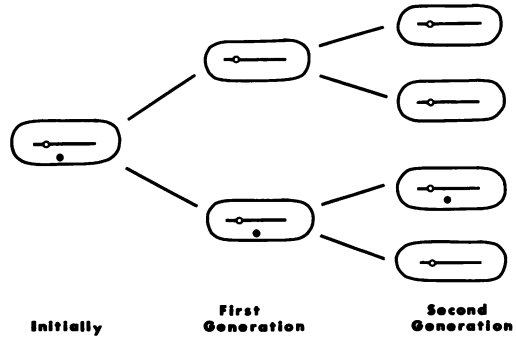

FIG. 10. *Distribution of a nonreplicating genetic element among dividing bacteria. The closed circle is the nonreplicating element; the open circle and line represent the replicating genome. At each generation, the fraction of cells containing the marker decreases by one-half. Based on Meynell (40).*

major factor in loss of viable cells from the intestine, the ability to distinguish excretion from death is clearly of value. In most cases, the bacteria underwent only a few divisions in the intestine. If the mice had been treated with streptomycin to eliminate normal intestinal bacteria before inoculation (the infecting strain used being streptomycin resistant), cell division was more rapid. The results showed that the normal mouse intestine contained a mechanism which was bacteriostatic and weakly bactericidal to *S. typhimurium*.

In neither of the two systems described above did much bacterial division occur. In another study, Maw and Meynell (39) studied the growth in the mouse of *S. typhimurium*, a natural pathogen of this animal. The marker was a superinfecting mutant of phage P22. After intravenous inoculation the organism became lodged in the spleen. The viable count doubled every 24 hr, whereas the true doubling time as determined from rate of dilution of the genetic marker was 8 to 10 hr. This should be compared with the minimum division rate observed in the test tube of 0.5 hr. The division rate in the spleen was at best only 5 to 10% of the maximum observed in vitro, and the death rate in the spleen was quite low. These results show that the maximum potential growth rate is not reached in vivo even by an organism which is highly pathogenic.

Despite the fact that Meynell's method is restricted to organisms for which the relevant genetic combinations can be constructed, it is clearly an interesting one with considerable promise. It should be useful in many cases in which it is of value to measure the division rate of organisms newly introduced into a habitat. Its use in studies of host-parasite relations may give us new insights into mechanisms involved in the establishment of colonizing species.

METHODS ESPECIALLY SUITED TO STEADY-STATE POPULATIONS

A system in steady state is in a time-independent condition in which production and consumption of each element of the system are exactly balanced, the concentrations of all elements within the system remaining constant even though there is continual change. Steady-state microbial populations are found in a number of natural habitats: the rumen (31), the intestinal tract (21), hot springs (11), and perhaps even infected animals (23, 24). In the steady state there is no change in population size, even though cell division is taking place. Because of this, it is impossible to measure growth rates from changes in absolute numbers, and one must seek more ingenious methods.

As I have pointed out elsewhere (8), an important item in characterizing a specific steady-state system is its time constant. Although fluctuations in growth rate may occur over a short period of time, on a long view of the system these fluctuations can be ignored. Thus, in the system of decomposition which occurs on the deciduous-forest floor, there is a great change in nutrient input in the autumn when leaves fall, but on an annual basis the forest floor ecosystem is in steady state because no net accumulation of leaves occurs (44). In the rumen, great changes occur daily as a result of feeding, but in the long run the system is constant (31).

The chemostat is an excellent laboratory model of a steady-state population (5), and it is instructive to consider how one measures growth rate in a chemostat, the principles developed there being readily adapted to natural situations. Measurement of growth rate requires a knowledge of the volume of the system (v) and its flow rate (f). The turnover time is v/f and the doubling time is equal to the turnover time.

Let us consider how the principle of the chemostat can be applied to a natural system. In a well-mixed system such as the rumen, the problem reduces to one of determining the rate at which material passes through, that is, the flow rate f. Hungate (31) discussed this problem in detail. If a pulse of an inert material (the marker) is given at zero time and if mixing is completed quickly, the rate of change in concentration of this inert material will give a measure of the turnover time. Because of mixing of the marker with unmarked contents and with material entering later, not all the marker leaves during one turnover time, even though during this period the total material leaving equals the total entering. The half-time, that is, the time at which the concentration of marker reduces to one-half the initial, is equal to 0.69 of the turnover time. Suitable markers for use in the rumen include nondigestible materials from the feed such as lignin or silica, or artificial markers such as iron oxide, chromic oxide, polyethylene glycol, rubber, etc.

If we know the turnover time (flow rate), and we know that the microbial population is in steady state, we know the doubling time of the population. To calculate the *productivity* of the population, that is, the number of new cells produced per day, we must also know the absolute population size, which can be determined by direct microscopic count. In the sheep rumen, there are about 10^{10} bacteria per ml and since the average volume of the sheep rumen is 5 liters, the total population is about 5×10^{13} bacteria. If the turnover time were 24 hr, 5×10^{13} bacteria would be produced per day.

In this discussion it has been assumed that bacteria disappear from the system only by passing out of it. If predation or lysis occurs within the system, the estimate of productivity could be low. Further, the estimate is for the rumen as a whole and assumes that it is a completely mixed system. It is quite possible that there are microhabitats within the rumen (e.g., the surfaces of food particles) where growth is rapid and other habitats with reduced ability to support microbial growth, so that the technique does not necessarily measure the maximum potential growth rate possible for rumen microorganisms. If for some reason knowledge of this potential were needed, one of the techniques described earlier in this paper, such as measurement of the rate of development of microcolonies, would be necessary. Although this has not been specifically done for the rumen, it could probably be done relatively easily (R. Hungate, *personal communication*).

If measurement of flow rate is not possible, growth rate in a steady-state system can be determined from the rate at which organisms leave the system, assuming that all organisms produced within the system are excreted intact and can be collected and counted. If the total number of organisms in the system is also known, the growth rate can be calculated. With this technique, neither the volume nor the flow rate needs to be measured, but the system must be completely mixed. This method was used by Gibbons and Kapsimalis (21) to measure the growth rate of the total intestinal microflora of hamsters, guinea pigs, and mice. The animals were placed in individual cages containing coarse-screen bottoms, and the fecal pellets which dropped through were collected. Twice a day for 3 days the pellets were collected, weighed, and homogenized in a diluent, after which direct microscopic counts were performed. Estimates of the total quantity of bacteria excreted per day were made by multiplying the number of bacteria per gram of feces by the average number of grams of feces excreted per day. After the excretion rate was determined, the animals were sacrificed and the bacterial count of the entire intestinal canal was determined. The average number of generations per day was then calculated. The doubling times of the intestinal bacteria calculated in this way were fairly low, varying from less than one to about six doublings per day. The intestinal flora of mice seemed to grow faster than those of hamster and guinea pig, but for all three species of animals the rates were much lower than those of typical intestinal bacteria in laboratory culture.

One possible limitation of this experiment is that bacteria might be destroyed within the intestine by predation, parasitism, or lysis. To check this, Gibbons and Kapsimalis (21) repeated their experiments with gnotobiotic mice inoculated with a pure culture of *E. coli*. Even under these presumably ideal conditions, *E. coli* grew at average rate of only 1.2 divisions per day. One explanation for this observation may be that environmental conditions (e.g., nutrient availability, *p*H, presence of natural inhibitors) may be inherently unfavorable for more rapid growth. Another possibility is that in certain microenvironments within the intestinal tract the growth rate is much higher than for the intestinal tract as a whole. It seems to me that the latter explanation has considerable merit, since the intestinal tract is highly heterogeneous both physically and chemically, and it is well established (51) that high bacterial populations live attached to the walls of the intestinal tract. If all the bacteria are growing in microenvironments which occupy only 10% of the total intestinal volume and after being sloughed into the lumen grow no further, the growth rate in these microenvironments would be 10 times that of the intestinal tract as a whole. Furthermore, in these experiments the assessment of total number of cells per intestinal tract included both growing and nongrowing cells. If all the cells were produced in 10% of the habitat and after entering the rest of the habitat grew no further, they would still be counted in the assessment of total numbers and would enter into the overall calculation. For instance, if the total bacteria excreted were $1{,}000 \times 10^9$ and if the count for the whole intestine were $1{,}000 \times 10^9$ but only 10% of the cells were from growing habitats, then the growth rate would be $1{,}000/100 = 10$ generations per day rather than 1. This serious error could be eliminated if only the cells in growing habitats were counted, although such counts could be done only if it were known where growth was taking place. Autoradiographic methods might provide a solution to this last problem.

Another approach to the measurement of steady-state growth was taken by Brock and Brock (11) for use in their studies on thermophilic algae in hot-spring drainways. It involved measurement of the algal wash-out rate after growth was prevented by darkening the system. Imagine a chemostat operating under steady-state conditions but with unknown volume and flow rate. In principle, the generation time could still be determined if growth were completely inhibited in a manner which did not otherwise alter the system. Wash out would still continue and the wash-out rate could be estimated by performing cell counts at various intervals of time. Since

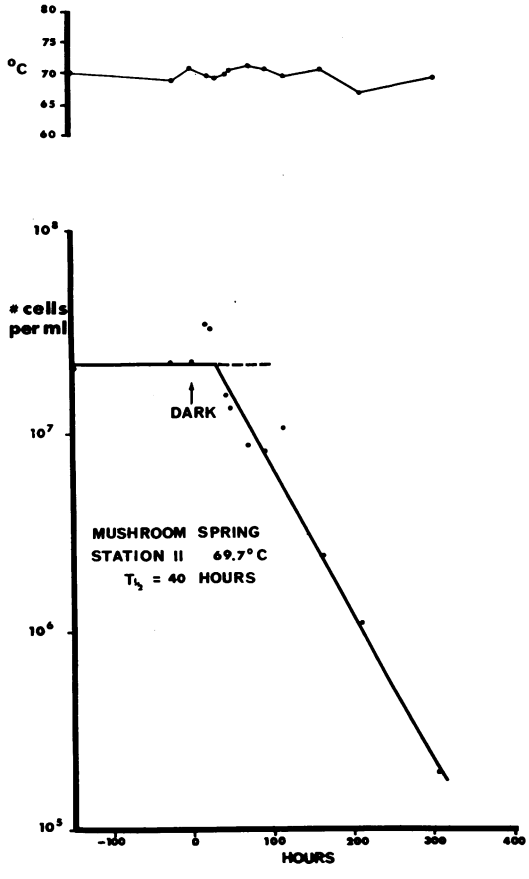

FIG. 11. *Rate of loss of algal cells from a darkened portion of a hot-spring algal mat. From Brock and Brock (11).*

growth balances wash out, the growth rate could then be determined.

This approach was relatively easy to use in hot springs because the effluent channels provide flow rate and temperature characteristics that are relatively constant over extended periods of time and because the algal population which develops reaches a steady state when the thickness of the mat is such that self shading occurs. Water flowing across the surface of the mat causes erosion of the cells through mechanical action, and the lost cells are replaced by newly grown ones. In areas of laminar water flow, a uniform mat develops from which replicate samples can be taken at different time periods. In studies at Mushroom Spring extending over 3 years (7, 11, 12), population density at selected stations remained fairly constant. However, if the system was darkened the algae disappeared quickly and were virtually absent within 2 to 3 weeks.

The results of a typical darkening experiment are given in Fig. 11. It can be seen that within 2 days after darkening the population density begins to decrease and that the rate of loss is exponential over at least two decades. In the example of Fig. 11, the half-time of the rate of loss was 40 hr. If the assumptions made above are valid, the doubling time of the population in the steady state was 40 hr.

During the first day or two after darkening there is virtually no loss of cells. Perhaps this lag reflects normal adaptation of the algae to alternating day and night, wherein during the day products of photosynthesis are stored for use as energy sources during the night (12). Further, although cell division may occur only during the night, as in *Chlorella* (54), this does not affect the calculation of the doubling time of the *population*. Finally, for the technique to work the mechanism by which the cells are lost from the system does not really matter; erosion, lysis, or predation could be responsible. (In a system in which cells die but do not disappear, viable counting or autoradiography might be used to measure loss rate.)

In the hot-spring system it was possible to show directly that cell loss was due to erosion in the following way (*unpublished data*). Temperatures around 70 C are near the upper limit for blue-green algal growth, so that water flowing over the mats comes from alga-free areas. Large volumes of water were collected just downstream from stations where darkening experiments had previously been done (the mats being first allowed to return to the steady state), and these water samples were passed through membrane filters to remove the algal cells. The algae were then counted on the membrane filters by using vertical illumination on a fluorescence microscope, the algae being recognized because of the intense red autofluorescence imparted by chlorophyll and phycocyanin. In this way, the density of algal cells in water which had flowed over the mat could be calculated. This calculated erosion rate was similar to the loss rate measured in the darkening experiments, thus providing evidence that cell loss in the steady state is due to erosion rather than to lysis or predation.

Another factor to be taken into consideration is the error which might arise if cells from water settled under the darkened area. If the mat under study receives water from an upper algae-containing area, since such water contains algae, the loss rate would be underestimated if settling occurred. This point was checked (*unpublished data*) by placing glass slides containing depression wells on top of portions of algal mats under the black covers. Some algal cells did settle onto the

slides and, at intervals, slides were removed and algal densities were quantified. The study showed that settling rate was two to three decades lower than wash-out rate, so that this factor was not a material source of error. Apparently, once the cells are taken into the rapidly flowing water their chances of settling are poor until the water has reached the foot of the spring, at which flow rate is much slower. In other habitats, settling may be more significant; thus, controls for settling should always be used.

It should be emphasized that the steady-state darkening technique measures only the growth rate of the population as a whole. It is possible (in fact, likely) that growth rates of different subsets of the population may vary quite markedly from this average value. In these compact algal mats in which self-shading is considerable, cells in the deeper parts of the mat receive much less light than do surface cells. Quantitative grain counts of autoradiographs of mats labeled with ^{14}C from CO_2 showed a progressive decrease in label from the surface to the bottom of the mats, which were 5 mm thick (12). Thus, although in the study presented in Fig. 11 the population as a whole was doubling every 40 hr, the cells at the surface were photosynthesizing faster and were probably developing faster than those at the bottom. This is, of course, the same problem which we discussed earlier in relation to measurement of growth rate in the rumen and intestinal tract. In terms of what the organisms are doing to the ecosystem, it is the growth rate of the whole population which is of interest. If one wishes to determine the fastest growth rate which an organism is capable of realizing in nature, another technique will be necessary.

The measurement of wash-out rates in growth-inhibited, steady-state populations can be applied with suitable modifications to other systems, both photosynthetic and heterotrophic. With photosynthetic systems, removal of the energy source without disturbing the system is relatively easy and could be applied to other algal habitats such as rivers, soils, and rocks. (The darkening of a whole lake would probably be beyond the capabilities of most research studies.) It is important to know in such studies that the factors affecting wash out are not themselves altered by darkening. For instance, if loss of algae was due not to erosion but to animal grazing, it would be necessary to ascertain that the animals involved were not themselves affected (physiologically or behaviorally) by darkening.

With heterotrophic systems, the inhibition of growth would probably require the use of an antibiotic which was specific for the organism of interest but did not affect other activities of the system. For studies of bacterial growth in higher animals, a whole array of suitable antibiotics is already available. For instance, to study the growth rates of intestinal organisms, antibiotics such as streptomycin and neomycin, which do not pass through the intestinal wall into the blood stream, could be easily used. It is well known that administration of such antibiotics orally results in inhibition of growth and elimination of intestinal bacteria. Although these antibiotics have been used both experimentally and clinically to sterilize the intestine, they have not been used in quantitative measurements of the rate of wash out of bacteria from the intestines.

One study involving the growth of *Mycobacterium tuberculosis* in the lungs of mice is relevant to this discussion. Gray and Cheers (24) showed that after an initial establishment period which took 6 weeks, the *M. tuberculosis* population reached a steady-state level in the lung which was maintained for at least the next 12 weeks. If infected mice were treated with drugs (pyrazinamide plus isoniazid) after the steady state had become established, there was a prompt exponential drop in numbers with a half-time of less than a week. This interesting experiment was done for another purpose than measuring growth rate, and because only viable counts were made it is not clear whether loss is due to lethal effects of the drugs or to destruction of cells by the animal after growth had been inhibited. In the latter event, these data could be used to calculate the steady-state growth rate.

Even if a population is not in steady state, growth rate might in principle still be measured. In a habitat in which absolute cell numbers are increasing, losses will still occur through predation, lysis, grazing, or wash out. Under these conditions, loss rates could be measured by a growth-inhibition procedure for samples taken at different times, and the loss rates obtained at each time could then be added to the observed rate of increase at each time, thus making possible a calculation of the actual growth rate. Admittedly, this technique is laborious.

A stable population size does not necessarily indicate a steady state in which growth is occurring. The organism may be in a habitat in which grazing, lysis, and wash-out losses are essentially zero but in which conditions are unfavorable for growth. A situation of this kind was found by W. N. Doemel (Ph.D. Thesis, Indiana University, 1970) in his studies on the growth rate of the eucaryotic alga *Cyanidium caldarium* in acid hot springs. Artificial channels placed in the flowing water provided fresh habitat for coloniza-

tion and growth by *C. caldarium*. After an extended exponential increase in cell numbers, the population reached a constant level. Darkening of a portion of the channel at this time did not result in immediate wash out, although colonization did not occur on a fresh darkened channel. The constant population level reached by the organism thus resembles a stationary phase rather than a steady state. The probable explanation is that in its niche there are rarely predatory or grazing organisms, and, because the cells are quite dense and the flow in the acid streams is not very turbulent, erosion of cells from the mat by the mechanical action of the water is minimal.

Another method for measuring growth rate in the steady state is to inhibit wash out, grazing, or other losses and to measure the rate at which the population whose density was kept in check by one or more of these factors increases after the check has been removed. This presupposes that the limiting factor can be eliminated without altering the system in other ways. Gambaryan (20) used this method to measure generation times of bacteria in muds of Lake Sevan (U.S.S.R.). In this habitat, bacterial growth took place primarily in the liquid phase, and protozoa or other animals feeding on the bacteria could be eliminated by passing the muddy water through filter paper. Controls showed that these filtrates did not differ significantly in physiochemical respects from the original muddy water. To obtain an estimate of the generation time in mud, bottom samples were filtered and the filtrate was divided into two samples; one of these was used to obtain the initial bacterial count and the other was placed in a test tube which was sealed and immersed in the lake at the sampling site. After 24 hr, the test tube was removed, samples of the initial and final samples were passed through membrane filters, the organisms were stained on the filters, and microscopic counts were performed. Generation time was calculated from the formula: generation time = (time of incubation) (log 2) divided by (log final count minus log initial count). In various parts of the lake, generation times of 10 to 281 hr were determined. Unfortunately, controls to show that the population density was being kept in check by protozoa and other animals which consume bacteria were not included.

El-Shazly and Hungate (18) used an analogous approach to measure net growth rate of rumen microorganisms. The population densities of samples of rumen material were assessed immediately after removal and again after incubation in vitro for 1 hr under simulated rumen conditions. The relative population densities were estimated by making maximal fermentation rate measurements; thus, rates of change in population size could be determined. It must be assumed that the only role of the glass bottle is to confine the growing population and prevent wash out and that secondary changes do *not* take place during the 1-hr incubation. To check this, studies were also carried out on samples incubated in dialysis bags in the rumen of a fistulated animal; the data obtained were similar to those obtained in vitro. It must also be assumed that losses due to protozoal grazing are negligible. In these studies, the net growth values per hour ranged from -7 to $+27\%$ of the population, but even in a single animal marked differences were observed over a diurnal feeding cycle. Net growth was smallest (or even negative) just before the morning feeding, when bacterial nutrients were probably limiting; the highest growth rate was found several hours after feeding. By adding growth rates obtained over the diurnal cycle, a daily growth rate (turnover time) of 1.92 was obtained, in good agreement with estimates made from calculations of wash-out rates from use of nonmetabolizable tracers to measure dilution rate.

Experiments of this type could, in principle, be done in any habitat in which losses due to grazing are low or in which grazers could be eliminated. However, since incubation time must be kept very short to avoid secondary changes in the bottle, accurate methods for assessing population size are necessary to permit detection of small differences. An incubation time less than one-tenth the doubling time is probably desirable. In phytoplankton studies, population densities can be assessed very accurately by use of $^{14}CO_2$, making it possible to use this method fairly easily; Eppley (19) has applied it with some modifications to measurement of growth rates of phytoplankton living in the sea off La Jolla, Calif. (The study was actually designed to measure the standing crop of photosynthetically active phytoplankton, but it determines growth rate as well.) Samples of seawater were filtered through 150-μm netting to remove larger animals and were then incubated on shipboard under in situ light and temperature conditions with $^{14}CO_2$. Samples were taken at 24 and 48 hr, and the assimilated radioactivity was determined by membrane filtration. From changes in rate of ^{14}C uptake after 24 and 48 hr, the specific growth rate (k) was calculated from the equation k = 1/days ln (48 hr-uptake $-$ 24-hr uptake/24-hr uptake). Apparent growth rates of about one to two doublings per day were obtained. The incubation times which Eppley used were long, but were so chosen to avoid problems associated with diurnal periodicity in algal photosynthesis. Shorter incubation times could be used if high specific radio-

activity $^{14}CO_2$ was used and if periodicity was obviated by incubating under saturating light to obtain the potential maximum photosynthesis rate. With such modifications, the method becomes precisely analogous to that of El-Shazly and Hungate (18).

INOCULUM SIZE: RATE OF EFFECT

An interesting technique quite different from any of the above has been used to estimate the growth rate of the spirochete *Treponema pallidum* in syphilitic lesions in the rabbit (17). An average incubation time of 17 days is required for a demonstrable lesion after the intradermal injection of 500 organisms; and the time required for the development of a lesion is reduced by 4 days for each 10-fold increase in inoculum size. Since a 10-fold increase in number is equivalent to 3.3 generations, the generation time of the spirochete in vivo can be estimated as 4 days/3.3 generations or 1.2 days (29 hr) per generation. It is assumed that each inoculated cell is capable ultimately of producing a lesion.

With modifications, this technique might be applicable to various other situations (in, for example, nodulation rate by rhizobia or the rate of establishment of a component of the intestinal flora).

CONCLUDING STATEMENT

A wide variety of methods for measuring microbial growth rates in nature is available. At least one of the methods described should be applicable to any specific ecological situation.

Is the result obtained worth the effort? This can be answered only in the context of a particular study. In a large ecosystems study, the unavailability of information on microbial growth rates would be a serious lack. In a simple study of the physiological ecology of a single organism, the knowledge of growth rate may be a useful parameter for the interpretation of the response of an organism to environmental variables. In our own studies on the ecology of *L. mucor* (33) and thermophilic blue-green algae (11), a knowledge of growth rates provided an important insight into how these organisms succeed in their natural habitats. Such information is of considerable value in interpretation of evolutionary processes, and it significantly supplements the conclusions drawn from studies of the same organisms in laboratory systems. Indeed, without such studies in nature, the laboratory studies are not too useful.

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