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Natural Selection in the Microbial World

The Second Marjory Stephenson Memorial Lecture

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It will be unnecessary, I trust, to dwell upon my gratitude to the Society for inviting me to deliver the second lecture in memory of Marjory Stephenson. I realize that my good friends in England are responsible for initiating and supporting the move that led to the final invitation, and that I owe this honour largely to their efforts.

The choice of an appropriate subject has been very difficult. This was mainly the result of having talked myself out during an extended lecture tour in the United States last year. After much contemplation and consultation I decided on a discussion of natural selection in the microbial world, partly because the topic holds a great deal of fascination and promise, partly also because it represents the other extreme end of the spectrum of research activities in microbiology that Marjory Stephenson had outlined in 1945 (Woods, 1953). It deals with the behaviour of micro-organisms in mixed cultures under natural conditions, and with the factors operative in the struggle for survival.

The general microbiologist recognizes the existence of a vast variety of micro-organisms, differing from one another in morphological respects, in developmental history, or in physiological and biochemical characteristics. To the scientific mind this very fact poses two fundamental questions. One is concerned with the manner in which this diversity came about; the other with the problem how the numerous types can maintain themselves in nature. I believe that at present there is no valid reason for thinking about this situation otherwise than in terms of the concepts developed, about a century ago, by Charles Darwin to account for the occurrence of the multitude of higher plants and animals. The main feature of the Darwinian approach is the introduction of the principle of natural selection. It implies that the environmental conditions determine which types can best compete with others, and thus survive. Granting the presence in a population of individuals with different characteristics, Darwin thus suggested a mechanism for the persistence of the 'fittest'.

That this mechanism also operates in the microbial world has been amply illustrated by many pertinent investigations which have, furthermore, provided information concerning the nature of specific factors involved in the selection process. It is the intent of the present paper to review some aspects of these studies. I shall not discuss the manner in which differences may originate among the individuals of an initially homogeneous microbial population. This is all the more unnecessary because an important contribution to this phase is supplied by the symposium on 'Adaptation in Micro-organisms',

organized by your Society a few years ago. Suffice it to say that two fundamentally different processes have been found to occur, in protozoa, algae, fungi, yeasts and bacteria, resulting in the appearance of variants. They are: (1) adaptations, in the sense of changes of properties in response to environmental influences, without affecting the genetic composition of the organisms; (2) mutations and hybridizations, whereby the genotype is modified. It seems reasonable to assume that the latter processes have been largely responsible for the appearance of progressively more aberrant types, and in this manner have supplied the material upon which natural selection could act.

Many types of micro-organism appear to have persisted in nature for a comparatively long time. This follows from the fact that they can be found to-day, and recognized as identical with microbes encountered and described decades or even centuries ago. We may thus conclude that there must exist ecological niches which permit such organisms to maintain themselves in competition with others. In addition, the particular ecology of a specific microbe must differ from that of all others, and the requisite conditions must, from time to time, be realized in nature. Otherwise the organism would eventually disappear completely. It is one of the pious hopes of the medical profession that this situation might be accomplished with respect to some of the pathogenic micro-organisms, although there is also evidence to the effect that natural selection may counteract efforts in this direction.

Ecological studies aim at supplying the data on which to base an interpretation of the manner in which environmental factors determine the outcome of the struggle for existence. Progress in this direction has been very slow for the higher plants and animals; in most cases it has not been possible to reach conclusions extending beyond those of a very general nature. One may, for instance, explain the barrenness of certain areas as a result of the lack of sufficient moisture to enable plants to grow. And since animals are, directly or indirectly, dependent on plants as their ultimate source of food, the absence of animal life can thus also be accounted for. But investigations of a more refined nature have only recently been initiated, especially in the completely air-conditioned greenhouses at the California Institute of Technology, under the direction of F. W. Went. The results so far obtained indicate how much more may be expected from an extension of this work. To mention a few illustrative examples: the peculiar property of the seeds of certain desert plants to germinate only after a second rainfall of a definite magnitude is currently attributable to the presence in the seeds of substances which inhibit germination. Not until these have been removed by adequate leaching can the seeds sprout. This behaviour ensures that at the time of germination the water-level in the soil will be sufficiently high to permit further development to the point of fruition. Seeds that germinate after the first moistening cannot develop to this stage, and plants that behave in this manner are doomed to extinction in the desert environment.

A similar situation has been discovered in connexion with temperature and light. Some higher plants can complete their life cycles only if during the night the temperature regularly falls below a definite maximum; others if the

duration of the periods during which they are exposed to light and darkness bears a definite relationship. The inability of such plants to flower and fruit under different circumstances tends to restrict their natural distribution to regions where the requisite conditions are met. Even though the physiological and biochemical events that govern flower- and fruit-formation may not be understood as yet, the results mentioned indicate the direction in which future research should move to reach a better comprehension of the regulatory mechanisms, and hence of the ecological relationships.

In studies on animal ecology, too, an occasional glimpse of salient environmental factors has been gained. An illustration is provided by the studies of Dobzhansky on the correlation between special environmental factors and the distribution of geographically distinct races of fruit flies. In collaboration with Mrak and El Tabi it has been established that different races of *Drosophila* display marked differences in their preference for specific yeasts, and that the occurrence of the latter is determined by the types of plants found in the environment. Thus a hitherto unavailable clue has been discovered which is apt to lead to a better understanding of *Drosophila* ecology.

Unfortunately, the relationships between the characteristics of an environment and the flora and fauna found therein must often be deduced from observations made at a time when the organisms are already present in large numbers. This is not always a satisfactory guide to an interpretation of ecological factors, because at such a time the environment may have been considerably modified by the activities of the organisms themselves. A simple case may illustrate this.

In many instances the growth of phytoplankton in a body of water is limited by the supply of minerals, particularly nitrogen compounds and phosphate. Since these substances are removed at an accelerated rate as the number of organisms increases, it stands to reason that at the time of maximum plankton growth the mineral content may be at a minimum. It would obviously be a fallacy to conclude from the chemical composition of the water at this time that absence of minerals is the cause of plankton development. In view of present knowledge, it is unlikely that anyone would confuse the cause-and-effect relationship in this case. But the situation is not always so simple.

It follows that a more dependable approach calls for observations prior to, as well as during, the appearance of specific organisms, so that an accurate picture of the sequence of events can be obtained. In this manner ecological relationships may be inferred with less ambiguity, and these can be subsequently tested by special experiments. Instructive in this connexion are the studies of Barker on the succession of phytoplanktonic components in marine bays (Barker, 1935). Fertilization of the sea water with minerals through land drainage or upwelling frequently causes the appearance of an abundant diatom flora, which is superseded by the development of dinoflagellates in equal profusion. The regularity of the sequence—relative paucity of phytoplankton, fertilization, appearance of diatoms, and, finally, of dinoflagellates—suggested that the growth of the diatoms so modifies the environment that it becomes more suitable for the competitive growth of dinoflagellates. One

important factor determining the succession is the decrease in the concentration of nutrients during the diatom bloom. Experiments with pure cultures have revealed that diatoms grow more rapidly than dinoflagellates as long as the concentration of nitrogen compounds and phosphates exceeds a certain value. Below this level the growth rate of dinoflagellates can be the greater; in fact, these organisms develop with undiminished vigour at concentrations some hundred times lower than those needed for an optimal rate of diatom multiplication.

Although simple ecological observations may sometimes be difficult to interpret unambiguously, a satisfactory choice between alternatives may often be reached by a judicious extension. A striking case in point is furnished by the ecology of the sulphur bacteria. These organisms are encountered in environments containing hydrogen sulphide, and many of them can be easily recognized because they contain conspicuous inclusion bodies in the form of sulphur droplets. For many years the ecological relationship had been interpreted to mean that the sulphur bacteria are the cause of the hydrogen sulphide production. This view was supported by the copious liberation of hydrogen sulphide when masses of sulphur bacteria are stored in the laboratory. Nevertheless, an opposite inference was theoretically possible, and Winogradsky, aware of this possibility, carried out his masterly experiments which eventually led to the revolutionary concept of chemo-autotrophy (Winogradsky, 1887, 1888*a*). By showing that the growth of the sulphur bacteria is dependent on the presence of hydrogen sulphide, he finally resolved the ambiguity of the ecological situation. And, though it must be admitted that the novel interpretation was proved by straightforward physiological experiments, it should here be emphasized that ecological studies played a significant role. For as soon as the alternative explanation had been conceived, Winogradsky realized that it implied a verifiable consequence; if sulphur bacteria grow in a special environment because it contains hydrogen sulphide, the organisms should also be found in places where this constituent is present as a result of non-microbiological activities, such as in sulphur springs. The inference was tested and found to be correct; sulphur bacteria are found in all sulphur springs.

But once the ecological situation had thus been clarified, a new problem had to be faced. The fact is that there are many different types of sulphur bacteria, and this, in turn, implies that these types must require special conditions, different for each one, for their continued existence. Hence it becomes necessary to define these conditions in order to provide the basis for a better understanding of the mechanism of natural selection in this group of micro-organisms.

A beginning has been made in this respect. The sulphur bacteria can be divided into two distinct groups, comprising the coloured and the colourless representatives, respectively. The former are anaerobes and depend on a supply of radiant energy as well as of hydrogen sulphide. They can therefore develop only in places where light is available and where the sulphide concentration is maintained at a sufficiently high level to preserve anaerobic conditions. Moreover, in this group a further ecological segregation occurs under the

influence of differences in sulphide concentration. In crude cultures the green sulphur bacteria consistently develop closer to the region of hydrogen sulphide production than do the red and purple sulphur bacteria, which indicates greater tolerance for the toxic substrate on the part of the first-mentioned organisms. Differences in tolerance for hydrogen sulphide exist even among the representative types of the red and purple sulphur bacteria, and it is thus reasonable to invoke concentration gradients as an important factor governing the distribution, and hence the natural selection, of the individual types. It may here be mentioned that these conclusions have been substantiated by the outcome of experiments of a sort to be discussed presently, viz. with elective cultures (van Niel, 1931; Larsen, 1952, 1953).

Much remains to be done, however, before we shall be in a position to specify accurately the conditions under which each one of the many readily distinguishable types can maintain itself in nature. At present we are, for example, completely ignorant of the factors that permit the occasionally observed mass development of the red sulphur spirilla. A more complete knowledge of their ecology should go far towards solving the problem of culturing these interesting organisms; as far as I know, not a single representative of this group has yet been isolated in pure culture, nor has it been possible even to propagate them in the laboratory in crude cultures.

Still more difficult is the situation concerning the colourless sulphur bacteria. Despite the fact that the conspicuous filamentous forms, *Beggiatoa* and *Thiothrix*, are often encountered in nature, we know little about the environmental factors that govern their occurrence, except that they are found in places where hydrogen sulphide and oxygen are present. The tantalizing *Thiovulum*, too, has long been refractory to more detailed studies, although Dr Wijler's recent experiments bring a glimmer of hope that the picture will be different in the not too distant future (unpublished; personal communication).

Ecological considerations long ago led Winogradsky to the conclusion that the brown, ferric hydroxide-encrusted bacteria of the *Leptothrix* and *Gallionella* groups represent ecotypes dependent on a supply of ferrous iron as the main oxidizable substrate (Winogradsky, 1888*b*). All the subsequent work on the distribution of these organisms has supported the contention that they occur only where reduced iron compounds are continuously available. Nevertheless, the natural environment has not yet been characterized accurately enough to permit further inferences, and the question whether they represent potential chemo-autotrophs is still an open one.

As has been indicated, the most serious difficulty in drawing conclusions from ecological relations is that a satisfactory interpretation must be based on a knowledge of the conditions leading up to the appearance of a particular organism. In this connexion I cannot refrain from mentioning some cogent remarks, made a few years ago at a meeting of American bacteriologists. Eklund, the director of a group of scientists studying the factors responsible for the outbreak of the dreaded poliomyelitis disease, stated that in his opinion the programme is greatly handicapped by the fact that the group is summoned

to a particular locality at a time when the disease is already in evidence. It is, unfortunately, not yet possible to predict when and where an epidemic may strike. This precludes the possibility of making on-the-spot observations prior to the appearance of the first cases. It must, of course, be realized that the alternative to the present approach would be to establish a laboratory for polio research at a more or less arbitrarily selected place where the group could assemble data on environmental conditions, on the off-chance that at some time an outbreak might occur there. Obviously, such an approach, though theoretically logical, would render the work most unsatisfactory for the investigators, because of the complete uncertainty of an outbreak occurring, and the establishment of such an institution cannot be particularly appealing to those who must furnish the funds.

These considerations show that the problems of natural selection and the factors governing it can be studied most satisfactorily if one can establish regularities in the appearance of a particular organism in a more or less reproducible environment. In that event one can determine the progressive changes in the latter before and after the appearance of the organism in question. It is not always necessary that the milieu be characterized in great detail; sometimes the factors responsible for the emergence of a special type of microbe can be surmised from observations on highly complex systems. A special case in point is furnished by the ecology of the previously mentioned colourless sulphur bacterium, *Thiovulum*. It is generally possible to obtain abundant cultures of this micro-organism by mixing a quantity of the marine green alga, *Ulva*, with some marine mud, placing the mixture in the bottom of a jar, filling this up with sea water, and incubating in darkness. Especially if a trickle of fresh sea water, entering near the bottom of the container, is constantly run in, *Thiovulum* here finds the ideal conditions for its growth, which is manifested by the appearance of a veil, often of quite irregular shape. Superficially, it suggests a typical stationary condition. Microscopic examination shows, however, that the veil is composed of a vast multitude of extremely motile ovoid microbes, stuffed with sulphur globules. The peculiar distribution appears to be the result of tactic movements by which the organisms maintain themselves in a near optimal situation. Further evidence to this effect is furnished by the observation that the shape and position of the veil is subject to continuous, though often small, alterations, and by the as yet unpublished studies of Dr Wijler. Guided by the assumption that, like other typical sulphur bacteria, *Thiovulum* grows only when supplied with hydrogen sulphide, which compound is produced during the decomposition of *Ulva*, he attempted to subculture *Thiovulum* under better defined conditions. This attempt has been crowned with success; it can be done by introducing a portion of the veil from a crude culture into a container filled with sea water, at intervals adding small amounts of hydrogen sulphide, and maintaining a continuous, slow current of fresh sea water, so that the jar is kept completely filled and overflowing.

These experiments have revealed a number of interesting aspects. In the first place, they have shown that physiologically *Thiovulum* is a typical sulphur bacterium. If addition of a fresh supply of hydrogen sulphide is post-

poned too long, the cells gradually lose their characteristic inclusion bodies, and soon afterwards perish. A timely supplementation with hydrogen sulphide causes the rapid reappearance of internal sulphur globules, as in Winogradsky's experiments with *Beggiatoa*. Ecologically more important is the fact that following a thorough stirring of the culture replenished with hydrogen sulphide, causing a more or less homogeneous dispersal of the organisms, a characteristic veil is re-formed within a matter of minutes, consistent with observations on the rate of movement of the cells, which is several hundred microns per second, and that the highest cell concentrations are found near the inflow of the fresh sea water. Another significant observation is that in a subculture not continuously flushed with sea water a surface film of other bacteria, probably representatives of *Thiobacillus*, develops. When this film forms, *Thiovulum* disappears.

It seems reasonable to infer from these results that the optimum conditions for growth of *Thiovulum* include a delicate balance between the oxygen tension and the hydrogen sulphide concentration of the environment. Deviations therefrom lead to overgrowth by other (sulphur) bacteria whose generation time is considerably shorter. In liquid cultures with a continuous flow of sea water *Thiovulum* can accumulate by chemotaxis in the most favourable regions; in stationary cultures the diffusion of oxygen into the medium is precluded by the bacterial surface film. It also seems possible that *Thiovulum*, one of the least-known sulphur bacteria, though described by Warming in 1875 and renamed by Hinze in 1913, may soon become available in pure culture as a result of an extension of Wijler's studies. In that case more detailed physiological experiments will become feasible, and these are necessary for an ultimate interpretation of the process of natural selection.

Nevertheless, however useful 'pure cultures' have been and will continue to be in microbiological work, they can no longer be considered as the eminently constant material which they once seemed to represent. Even in pure cultures variation and natural selection continuously take place, as demonstrated by the often disturbing fact that after a series of transfers to fresh media the resultant crop frequently appears to contain more than one type. The interpretation of this phenomenon has undergone many changes in the course of time, and it is the great merit of W. Braun (Braun, 1947) to have recognized that it can best be ascribed to the continuous appearance of mutants successfully competing with the parent type. We are still far from an adequate appreciation of the specific factors that govern such competition. It is true that Ryan and collaborators (Braun, 1953) have shown the existence of small but significant differences in generation time of wild-type and nutritionally-deficient mutants in favour of the latter, so that at least one of the mechanisms operative in their establishment can be appreciated. Nevertheless, not all mutants are or need be nutritionally deficient, and the highly artificial complex culture media so often used for the maintenance of pure cultures of micro-organisms hamper a more detailed analysis of the selective mechanism. Hence the main emphasis in what follows is concerned with ecological experiments in which numerous different types of microbes participate.

It was especially the work of Winogradsky on a restricted scale (Winogradsky, 1949), and of Beijerinck (Beijerinck, 1921) on a far more general basis, that has made the microbiologist familiar with the possibility of using a direct ecological approach to microbial ecology, by means of the elective, selective, or enrichment culture technique. Here a chemically and physically well-defined environment is chosen; it is inoculated with a sufficient amount of some naturally occurring material to justify the expectation that representatives of most microbial types are thus introduced; and regular observations are made on the microflora and microfauna that develop in the course of time. The outcome of the experiment indicates what organisms come to the fore; these are the ones that are obviously the 'most fit' to survive in competition with others. Such experiments are difficult to perform with larger plants and animals because of the need of large areas, the virtual impossibility of establishing uniform conditions therein, and the long time spans involved. In contrast, the microbes, with sizes of the order of magnitude of microns, permit the use of relatively small culture vessels; and the short generation times, usually measured in hours or fractions thereof, ensure the development of the 'fit' in numbers adequate for further study, often reaching densities of 10^9 individuals per ml. in the course of some days. Also the selection of an appropriate inoculum is simple, because experience has shown that an enormous variety of microbial types is present in a few grams of soil or mud, or in a few ml. of water from stagnant pools and ditches.

It is clear that every microbe in the inoculum capable of development in the environment supplied by the experimenter will multiply. In many cases the organisms that gradually compose the predominant population of such cultures are those whose minimum requirements are fulfilled. For an illustration we may once more draw upon the experience with the sulphur bacteria.

Characterized as autotrophic organisms that oxidize inorganic sulphur compounds such as hydrogen sulphide, they can grow in mineral media. The coloured, photosynthetic, representatives produce an oxidant photochemically; hence they do not require an external oxidizing agent, and can grow anaerobically when properly illuminated. The minimum requirements thus comprise an inorganic, sulphide-containing medium, devoid of an oxidant. And in such media only the coloured sulphur bacteria develop.

It is interesting to contemplate the fact that the environment must contain a special ingredient, hydrogen sulphide, over and above those needed for the growth of algae. Nevertheless, algae do not develop in the crude cultures until the sulphide has been utilized by the sulphur bacteria. The reason for this is that the necessary supplement is inhibitory to algal growth. Diatoms and green algae especially are very sensitive to sulphide; they are usually killed soon after introduction into the elective medium. But the blue-green algae, though much more resistant to the poisonous substance, are also inhibited as long as sulphide is still present. Thus the sulphur bacteria initially outgrow the Cyanophyceae, in spite of the fact that the minimum requirements of the latter are fewer than those of the former.

In this particular case another selective influence may also be introduced. This is the wavelength of the radiant energy. Photosynthesis of the pigmented sulphur bacteria, as of green plants, is accomplished with the aid of chlorophyllous compounds. But the absorption characteristics of the chlorophylls of the green and purple sulphur bacteria are both mutually different, and different from those of the algal chlorophylls; the bacteria display their maximum absorption in the infra-red region where the plant chlorophylls no longer absorb. It is therefore possible to supply cultures with radiant energy of wavelengths not effective for green plant photosynthesis without preventing the growth of the photosynthetic sulphur bacteria. That natural selection is often accomplished through this mechanism is shown by the fact that one may frequently observe mass developments of coloured sulphur bacteria underneath a practically solid layer of algae. And it is feasible to induce the preferential development of green or of purple sulphur bacteria at will by exposing elective cultures to appropriate regions of the spectrum.

The addition of yet another ingredient to the mineral sulphide medium in the form of an oxidizing agent permits the growth of colourless sulphur bacteria. These can grow in darkness where development of photosynthetic organisms is eliminated. The ability of *Thiobacillus denitrificans* to effect an oxidation of reduced sulphur compounds with nitrate as the sole oxidant permits this organism to grow anaerobically in nitrate-containing media; the other colourless sulphur bacteria appear to require free oxygen.

Another illustration of the operation of natural selection through the minimum-requirement mechanism is provided by the microbes that can use molecular nitrogen for the synthesis of proteins and other nitrogenous cell constituents. Long ago Beijerinck found that in strictly mineral media, free of nitrogenous compounds other than N_2 , blue-green algae usually develop in illuminated cultures inoculated with garden soil, water, or mud. This ecological observation led him to attribute to these organisms the ability to fix nitrogen. Beijerinck's conclusion was not generally accepted until 1928, when Drewes and Allison independently established this property beyond a doubt with pure cultures.

Large-scale natural selection by the absence of nitrogen compounds occurs around some of the hot springs in Yellowstone Park. Even in regions where the water is no longer warm, only blue-green algae are encountered in abundance. Inoculation of samples of this water with garden soil and incubation of the cultures in the light at room temperature also yields only blue-green algae, whereas the addition of nitrate or ammonium salts to the samples, leads to the development of green algae and diatoms, whose minimum requirements include the need for such substances.

If these observations, made in 1929, had been critically integrated with the noted presence of purple sulphur bacteria in Yellowstone hot springs, it should have been possible to infer the nitrogen-fixing ability of the last-mentioned organisms at that time. The fact that they possess this property was not discovered, however, until quite recently, and as a result of very different observations (Kamen & Gest, 1949; Lindstrom, Tove & Wilson, 1950).

Media devoid of combined nitrogen can also provide cultures of non-photosynthetic microbes capable of fixing nitrogen. For this purpose a supply of an energy-yielding substrate is required. The anaerobic *Clostridium pasteurianum* thus comes to the fore in sugar media incubated in the absence of oxygen; in aerobic cultures representatives of *Azotobacter* and *Beijerinckia* form the predominant microflora when the medium is supplied with any one of a number of simple organic substances. Theoretically, nitrogen-fixing bacteria that can live as chemo-autotrophs might exist, notably among the hydrogen-oxidizing types. So far no aerobic hydrogen-oxidizing bacterium with this property has been discovered; but Sisler & ZoBell have shown that *Desulfovibrio desulfuricans*, which can oxidize hydrogen with the concomitant reduction of sulphate to sulphide (Butlin & Adams, 1947; Senez & Volcani, 1951), may, in fact, fix molecular nitrogen (Sisler & ZoBell, 1951).

By the use of elective cultures it is thus possible to prevent the growth of a variety of microbial types present in the inoculum through the use of media of appropriate composition. Nevertheless, even though the principles and execution of this direct ecological approach are quite simple, the results of such experiments are not always easy to interpret. Difficulties are sometimes encountered; they stem, in part at least, from two sources. In the first place, it must always be realized that one introduces with the inoculum a certain amount of foreign material, and this inevitably modifies the environment. In the second place, the development of one or more organisms also results in a gradual modification of the environment, so that the altered medium may become more favourable for the development of other creatures.

The former difficulty can usually be overcome by the use of repeated transfers. In this manner the foreign material is eventually eliminated through dilution, and a more clear-cut relationship can thus be established between a defined environment and the microbes that preferentially develop therein. Occasionally this very procedure may, however, lead to results that are at first sight unexpected and puzzling. A good example of such a situation is furnished by recent investigations of Verhoeven and collaborators. In elective cultures for organisms that can oxidize hydrogen with nitrate in the absence of oxygen, a mineral medium was used and inoculated with garden soil. The first crude culture showed a copious development of *Micrococcus denitrificans*; but growth no longer occurred in transfers. Later it was found that for anaerobic development at the expense of hydrogen and nitrate *M. denitrificans* requires one or more growth factors. These can be provided by a trace of yeast autolysate; in the first culture they were evidently supplied by the soil sample used as inoculum (Verhoeven, Koster & van Nieuvelt, 1954). A very similar observation was made by Doudoroff in unpublished studies on aerobic hydrogen-oxidizing bacteria. The first crude cultures often contained an abundance of special types that could be perpetuated only in media supplemented with a trace of yeast extract. Another illustration is provided by the fact that elective cultures of *Azotobacter* tend to diminish in vigour as a result of repeated transfers because for growth in nitrogen-free media the organisms

require molybdenum and the soil inoculum supplements the sometimes inadequate molybdenum content of the solution.

A notable instance of an influence exerted by the growth of accompanying micro-organisms has recently been contributed by studies on the obligatorily chemo-autotrophic *Thiobacillus denitrificans*. This organism, discovered more than 50 years ago by Nathansohn and Beijerinck in anaerobically incubated mineral media containing thiosulphate or sulphur as oxidizable substrate and nitrate as the sole oxidant, appears to lose its activity in pure culture. A fairly recent attempt to re-isolate and study the bacterium (Syderius, 1946) was completely unsuccessful; the elective cultures contained so vast a preponderance of heterotrophic denitrifiers that only these bacteria were found on agar plates, a situation which persisted throughout repeated transfers. This is surprising because the composition of the medium is such that it would hardly be expected to support abundant growth of heterotrophs. Syderius suggested that the latter developed at the expense of organic substances synthesized by *T. denitrificans*; but, since this would require a production of organic matter in such profusion that the heterotrophs could outgrow the chemo-autotroph, the interpretation appeared rather improbable.

A different and more satisfactory explanation of the situation has come from the recent studies of the Baalsruds (Baalsrud & Baalsrud, 1954). They established that *Thiobacillus denitrificans* cannot grow at the expense of nitrate nitrogen which the organism readily and quantitatively reduces to molecular nitrogen. Hence it is understandable that nitrogenous substances, other than nitrate, are required for the synthesis of cell material. Indeed, the addition of an ammonium salt to the culture medium results in the copious growth of the denitrifying thiobacilli. Under these conditions the ratio of autotrophic to heterotrophic bacteria in the crude cultures is radically reversed, and the isolation of *T. denitrificans* presents no difficulties. From these observations it appears far more probable that in the cultures containing only nitrate as a nitrogen source growth of the thiobacilli is conditioned by the formation of NH_3 or some other reduced nitrogenous substance through the activities of heterotrophs. This implies that the latter must be present in reasonably large numbers to permit the growth of but a few of the autotrophic bacteria.

It is evident that one must be aware of complications such as those discussed above. Nevertheless, in many studies with elective cultures they are not likely to be encountered. As a rule, repeated transfers lead to cultures that become progressively more homogeneous—at least during their early phases. Later the situation usually becomes more involved because the development of the organisms primarily selected for by the environment causes changes in the medium. This, in turn, often leads to a more or less abundant growth of organisms that are particularly adapted to, and hence will be selected for, by the new conditions. A good example of such a situation is the appearance of large numbers of various protozoa in elective cultures of *Azotobacter* in nitrogen-free media, especially when the initial concentration of the oxidizable substrate has been low. This is obviously a typical case of a secondary development; it is improbable that anyone would consider the presence of amoebae

and ciliates in the cultures as evidence that these organisms use molecular nitrogen as their sole or even main nitrogen source. And microscopic observation of the protozoan behaviour reveals that they have developed because they can feed on the nitrogen-fixing bacteria which form the primary population of the culture. In general, it is not difficult to distinguish the later stages from the primary ones.

Occasionally, elective cultures can be established in which secondary developments do not occur. The best-known is that of anaerobic cultures of lactic acid bacteria in sugar-containing media. These organisms seem to sterilize the medium through the production of lactic acid in concentrations higher than those tolerated by any other microbe—with the exception of *Zymosarcina ventriculi*. The lactic acid bacteria are, consequently, the natural agents through whose activities various sugar-containing foodstuffs can be preserved by anaerobic storage. These organisms are among the most fastidious of bacteria; their nutritional requirements include a variety of B-vitamins and several amino acids. Media of a composition satisfactory for their growth are, of course, more than adequate for the development of many other and less exacting microbes. Nevertheless, the fact is that once again those microbes come to the fore whose minimum requirements are fulfilled, i.e. the lactic acid bacteria. And a simple reflexion will show that otherwise these bacteria would long ago have disappeared, whilst only the organisms with relatively simple needs would have survived.

To be sure, the mechanism whereby successful competition is here achieved is rather different from that discussed before. It may also be expected that additional factors, such as the production of substances that are toxic for potential competitors in very low concentration, as in the case of antibiotics, or differences in growth rate, as in the case of Ryan's nutritionally deficient coli mutants, will play a role in the 'struggle for survival' among micro-organisms. Unfortunately, little is known concerning these matters at the present time. For a better understanding of the problem of natural selection in the microbial world it is, of course, necessary to investigate each situation in detail.

It is also important that special attention be paid to some aspects of elective cultures that are sometimes glossed over. It cannot be denied that the use of such cultures has been instrumental in acquiring a better knowledge of those micro-organisms that predominate in a more or less well-defined environment. Furthermore, such cultures have often supplied the investigator with ideal experimental material for a detailed study of the degradation of a particular substance. By using this as the sole or main substrate in a culture medium, he can readily isolate organisms involved in its decomposition. Conversely, from such elective cultures much has been learned about the functions of various types of organisms in nature. It is a common enough experience that the same kind of bacterium, yeast, mould, or alga can be isolated in different places from similar elective cultures. And it is certainly logical to conclude from such results that also in nature the same type of microbe will be involved in the decomposition of the substrate in question. Sometimes it is thus found that an organism to which hitherto no special role could be ascribed turns up

regularly under specified conditions. A striking instance of such a situation is supplied by the recent studies on the bacterial oxidation of pyrimidines. Virtually simultaneously and completely independently, Hayaishi & Kornberg at Bethesda, Wang & Lampen at Cleveland, Lara at Pacific Grove, and Batt at Oxford, isolated the bacteria that develop in mineral media with pyrimidines as the carbon source. In each case the organism responsible for the decomposition of the substrate appeared to be a member of the *Corynebacterium-Nocardia* group (Hayaishi & Kornberg, 1951; Wang & Lampen, 1952; Lara, 1952; Batt, 1952). Although these bacteria had often been isolated from soil by direct plating on various media, so that their abundant occurrence in soils had been well established, a special ecological niche had not been assigned to them. It is now evident that they are the major, perhaps even the only representatives among the microbes that normally carry out the oxidative degradation of pyrimidines in nature in an otherwise mineral environment.

Experiences such as these are fully in keeping with the once widespread notion that the natural decomposition of many substances always occurs by means of highly specialized types of micro-organisms. Though this concept has its merits, it may also cause confusion if its implications are not clearly recognized. A number of studies on the aerobic decomposition of cellulose aptly illustrate this point. It was early recognized that this polysaccharide can be decomposed by many different fungi; but the bacterial degradation was believed to involve one particular organism, specialized to the point of being unable to use any substrate other than cellulose. When Kellerman, McBeth & Scales announced the isolation in pure culture of a considerable variety of cellulose-decomposing bacteria that could grow quite well in conventional, cellulose-free media, their claim that these organisms were among the causative agents of cellulose degradation in nature was challenged on the basis that the bacteria lacked the requisite degree of specificity. Also the later work of Gray & Chalmers, who had isolated a vibrio that can decompose agar as well as cellulose, was initially interpreted by Winogradsky as indicating that this microbe could not be 'the natural agent' concerned in either process, even if the claim for agar decomposition were sound. Nevertheless, in due course it was convincingly demonstrated that with the aid of elective cultures many different bacterial types can be obtained that are capable of aerobically degrading cellulose, and representatives of the *Pseudomonas-Vibrio* group, of the aerobic sporeformers, of the actinomycetes, and of the myxobacteria are now known to possess this property. Besides, this is not an isolated instance; in fact the number of processes for which but one single, specific microbe is responsible has dwindled almost to the vanishing point. Nor is there a lack of specificity only in this respect; it is an equally well-established fact that one and the same organism can decompose many different and chemically quite unrelated compounds.

That myxobacteria, pseudomonads, vibrios, actinomycetes, etc., appear in elective cultures with cellulose as substrate must, I believe, be taken to mean that under natural conditions, too, these different types are regularly or occasionally engaged in cellulose degradation. But, whilst their very persistence in nature argues eloquently enough for the recurrence of situations

causing their periodic natural selection, it would at present be rash to associate the presence of cellulose in an environment with the mechanism of the particular selection process. The outcome of elective cultures with cellulose as substrate is still wholly unpredictable. Perhaps this must be attributed to the fact that a mineral medium with a piece of filter-paper or cotton-wool, and inoculated with a few grams of soil, is anything but a homogeneous system. Thus the fortuitous presence on a soil particle in contact with a cellulose fibre of large numbers of an organism potentially capable of decomposing the polysaccharide would no doubt cause a localized development of this type, even though the factors normally causing its selection in nature may have little or no connexion with the presence of cellulose.

A comparable situation is presented by the outcome of parallel experiments in which, for example, acetate decomposition is studied under somewhat different conditions. On the one hand, a mineral solution with the substrate is inoculated with, say, 100 mg. of a soil sample; on the other hand, an equivalent quantity of soil is distributed over a culture plate containing the same medium solidified with agar or silica gel. After incubation for an equal length of time the microflora found in the two cultures is strikingly different. The liquid culture generally contains fluorescent pseudomonads to the exclusion of all other types; the plate culture is found covered with colonies of a great diversity of moulds and bacteria, with protozoa feeding on the other micro-organisms.

Such results seem to justify some deductions of importance for the problem of natural selection and the function of specific micro-organisms in nature. The outcome of the liquid culture suggests that the fluorescent pseudomonads are the effective agents for the mineralization of acetate. And no doubt they are. Yet, this conclusion seems contradicted by the cultures on agar, where such organisms are rarely encountered. This is in line with the common experience that pseudomonads do not constitute a quantitatively significant proportion of the soil microflora. The obvious answer to the dilemma thus created is that a completely aqueous environment favours the development of the pseudomonads so that even a single one of these organisms can outgrow competitors, while in soil special conditions cause the natural selection of quite different types for the execution of comparable decomposition processes. A similar experience has been gained from studies on organisms oxidizing hydrocarbons. Söhngen found that on mineral agar plates, inoculated with soil suspensions and exposed to hydrocarbon vapours, colonies of saprophytic mycobacteria appear in abundance (Söhngen, 1913). Later, Haag showed that all mycobacteria tested can oxidize hydrocarbons (Haag, 1927). But in liquid elective cultures, inoculated with the same soil samples, one generally finds pseudomonads as hydrocarbon oxidizers.

It is, therefore, clear that we cannot draw sound conclusions concerning the natural role of microbes from the experience gained with elective cultures unless we restrict our inferences to apply only to rigorously comparable conditions. This has been ably argued by Hungate in connexion with his studies on microbial cellulose decomposition in the rumen of herbivorous animals (Hungate, 1950). Inoculation of a mineral, cellulose-containing medium with rumen fluid, and incubation under anaerobic conditions may yield cultures of

cellulose-fermenting spore-formers. But this result merely shows that the inoculum contained such organisms, and not that they fulfil an important function in the rumen, where they may be present in very small numbers. After all, the rumen fluid is very different from a simple mineral solution, and it is only to be expected that in this environment other anaerobic cellulose-fermenting organisms will be selected for. We shall have to learn to interpret the normal occurrence of certain microbes in particular environments in terms that take account of special factors not ordinarily considered. We may find that the common association of pseudomonads, vibrios, spirilla, ciliates, and other highly motile organisms with bodies of water, and of microbes devoid of motility or exhibiting gliding movements, such as myxobacteria, blue green algae and amoebae, with soils or other largely particulate or solid media is the result of natural selection mechanisms that involve more than the mere provision of nutrient materials. It is probable that adsorption phenomena play an important role in creating or maintaining concentration gradients and ionic ratios.

That concentration of nutrients *per se* can influence the results is shown, for example, by experiments on the growth of freshwater algae in liquid elective cultures for which the same mixture of inorganic salts is used at different levels of concentration. In media with high concentrations unicellular green algae predominate; the lower the concentration, the greater is the proportion of filamentous forms developing from comparable inocula. The effect of special ionic ratios is evident from the fact that in solutions with a high magnesium content *Stichococcus bacillarus* tends to outgrow other algae. These illustrations may suffice to indicate a fruitful field for further investigations.

There are many microbes whose 'natural' functions are as yet unknown since they have not been encountered in elective cultures. A good example are the micrococci and sarcinae, so commonly found as aerial contaminants on agar plates. Here, some additional ecological data may help us better to understand the selective processes whereby these organisms manage to persist in nature. It seems to me suggestive that they are abundant on human skin, on cheese rinds, on sausage casings, and even in brine. In view of the complex nutrient requirements of the representatives studied to date, it may well be that they can outgrow potential competitors only in environments where the latter are inhibited by high salt concentrations.*

A curious representative of this group is the sporeforming, motile *Sporosarcina ureae*, first isolated by Beijerinck from liquid elective cultures in urea-containing media. Numerous attempts to re-isolate this organism by similar methods have failed completely. Nevertheless, it appears to be normally present in soil and can be isolated therefrom by direct plating on appropriate agar media, as shown by the extensive studies of Gibson, who also showed that in liquid cultures it is rapidly overgrown by urea-decomposing bacilli (Gibson, 1935). It should be interesting to test the effect of salt concentrations on the outcome of elective cultures.

* Dr B. A. D. Stocker has called my attention to a publication by Maitland and Martyn (Maitland & Martyn, 1948) which contains pertinent observations and cites earlier work that is fully consistent with this deduction.

The distribution of aerobic sporeforming bacteria in general presents a similar problem. Not a single member of this group is thus far known to thrive in media unsuitable for growth of non-sporeforming organisms. This suggests that the successful competition of the sporeformers is based on factors other than merely nutritional, and the conclusion lies at hand that these are associated with the formation of the highly resistant endospores. Thus they can survive under conditions that cause the destruction of non-sporeforming competitors. The trick of obtaining elective cultures of sporeformers by the use of pasteurized inocula is, of course, based on this principle.

It is true that our knowledge of the natural function of many micro-organisms is still fragmentary. But once an elective culture method for a particular microbe is available, it may be safely concluded that this organism will also be found in nature under conditions corresponding in detail to those of the culture, and that it will then carry out the same transformations.

Thus the elective culture techniques still offer great promise for improving our understanding of microbial ecology and of natural selection. Obviously, the choice of simplified environments for such studies also simplifies the interpretation of the results. It must, however, be recognized that in nature the conditions are seldom simple. Hence we must learn to study more carefully the effects of complicating circumstances, so that it may eventually become possible to realize in the laboratory those conditions that cause the appearance, distribution, and persistence of so many types in nature. This will require much imaginative work, and correlation of many kinds of observations. If we learn, for example, that the mushroom *Panus stypticus* is found in Europe as a non-luminous, and in North America as a luminous species; that the two forms are completely interfertile, and probably differ by no more than a single gene character, as shown by Ruth Macrae (1937); then it is clear that this distribution must be the result of natural selection. And one of the tasks of the general microbiologist will be to elucidate the manner in which such selections operate. It is a formidable task. Nevertheless, the microbiologist can take heart from the knowledge that the material he deals with represents life in a relatively simple form and can often be investigated under conditions far more favourable than those needed for similar studies on higher plants and animals, while yet the fundamental principles he may discover by his efforts will be applicable to all living organisms.

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