Thomas D. Brock

Thermophilic Microorganisms and Life at High Temperatures

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The figure on the frontcover is a photograph of the runoff channel of Grand Prismatic Spring, Yellowstone National Park, showing the extensive development of blue-green algae and photosynthetic bacteria.

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Preface

From 1965 through 1975, I conducted an extensive field and laboratory research project on thermophilic microorganisms. The field work was based primarily in Yellowstone National Park, using a field laboratory we set up in the city of W. Yellowstone, Montana. The laboratory work was carried out from 1965 through 1971 at Indiana University, Bloomington, and subsequently at the University of Wisconsin, Madison. Although this research project began small, it quickly ramified in a wide variety of directions. The major thrust was an attempt to understand the ecology and evolutionary relationships of thermophilic microorganisms, but research also was done on biochemical, physiologic, and taxonomic aspects of thermophiles. Four new genera of thermophilic microorganisms have been discovered during the course of this 10-year period, three in my laboratory. In addition, a large amount of new information has been obtained on some thermophilic microorganisms that previously had been known. In later years, a considerable amount of work was done on Yellowstone algal-bacterial mats as models for Precambrian stromatolites. In the broadest sense, the work could be considered geomicrobiological, or biogeochemical, and despite the extensive laboratory research carried out, the work was always firmly rooted in an attempt to understand thermophilic microorganisms in their natural environments. Indeed, one of the prime motivations for initiating this work was a view that extreme environments would provide useful models for studying the ecology of microorganisms.

As a result of this 10-year research project, I published over 100 papers. Although these were all published in reputable and widely available journals, the work has perforce been scattered. Because of a current widespread interest in thermophilic microorganisms, especially from the biochemical and evolutionary point of view, it seemed appropriate to summarize not only our own work, but that of others, with special emphasis on those organisms living under the most extreme environments. I hope that this book will not only be a useful reference to past work on these organisms, but will provide some insight into the directions future research might take, especially for field-oriented work. To this end, I have attempted to give detailed maps of well-studied Yellowstone thermal areas, and to provide good photographs of habitats that have been widely studied.

My own research work could not have been done without the collaboration of a large number of people. Because there may be some interest in the social history of a research effort such as this, I have provided a few pages of personal history in a terminating chapter, and credit is given there to the individuals who have been involved in this work. My own research has been supported financially by the National Science Foundation, the Atomic Energy Commission (subsequently the Energy Research and Development Agency and the Department of Energy), Indiana University, and the Wisconsin Alumni Research Foundation. Because of the faith they showed me at the inception of this work, I am especially grateful to the National Science Foundation, without whose initial support this research project would probably never have gotten off the ground.

May, 1978 THOMAS D. BROCK

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CHAPTER 14 (Omited)

Chapter 1
Introduction

There are several ways in which the work to be discussed in this book could be characterized. Studies that attempt to relate the characteristics of organisms to their ecology are best called *physiological ecology* and much of the work to be discussed in this book fits under this heading. Another major theme of this book is *biogeochemistry,* the study of chemical processes taking place in nature which are being carried out by living organisms. At one time, the whole body of work might have been called *geobiology;* indeed, the eminent Dutch scientist Baas-Becking once wrote a book with this title, which included the kinds of things I am going to discuss here. And finally, since much of the work deals with the structure, taxonomy, and biochemistry of microorganisms, it could (perhaps most aptly) be called *general microbiology.*

I was fortunate when my work began that a wide variety of techniques were available for the sophisticated study of natural ecosystems. Many of these techniques were not available to earlier workers in the Yellowstone habitats, which accounts in part for the tremendous amount of new information we were able to obtain rapidly. The most important technique was radioactive tracing, using ^{14}C, ^{32}P, ^{35}S, and ^{3}H. Counting by gas flow or liquid scintillation counting, or by microscopic autoradiography, made possible a wide variety of experiments. A Nuclear-Chicago gas flow detector and scaler was used for most work, but in later years we had a Beckman Beta-Mate liquid scintillation counter. I also had available a very good microscope, a Carl Zeiss Universal, with phase, fluorescence, and Nomarski optics. Spectrophotometers are not especially new, but had not been used in any previous Yellowstone work. We had a B and L Spectronic 20 for routine chlorophyll and chemical assays, and a Beckman DB-G spectrophotometer, with recorder, for scans. Temperature measurements in the

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field were made with Yellow Springs Instrument Co. thermistor probes and bridges, and over the 10 years we wore out four YSI bridges and innumerable probes. pH Measurements were made with Corning or Orion pH meters with combination glass electrodes. For one summer, we had a Packard-Becker model 419 gas chromatograph with flame photometric detector, for use in our sulfur work. Aside from these pieces of equipment, most of the work was done with simple glassware, plastic ware, test tubes, tanks of gases, immersion heaters for water baths and aquaria, etc.

Extreme Environments

What is an extreme environment? It is not appropriate to define it anthropocentrically, as we should be the first to admit that human life is not everywhere possible. More appropriate is its definition as a condition under which some kinds of organisms can grow, whereas others cannot. If we accept this definition it means that an environmental extreme must be defined taxonomically. Instead of looking at single species, or groups of related species, we must examine the whole assemblage of species, microbial and multicellular, living in various environments. When we do this we find that there are environments with high species diversity and others with low species diversity. In some environments with low species diversity we find that whole taxonomic groups are missing. For instance, in saline and thermal lakes there are no vertebrates and no vascular plants, although they may be rich in microorganisms and very high in the numbers of organisms of the species that do live there. In many of the most extreme conditions we find conditions approaching the pure culture, with only a single species present.

Such environments are clearly of enormous interest to the microbiologist, especially one interested in the ecological and evolutionary relationships of organisms. In such biologically simple environments experimental microbial ecology is most easy to carry out, since microbial interactions are minimized or absent. Compare the problems of the soil microbiologist, faced with thousands of species in a mere handful of dirt, with those of the thermal microbiologist, who can often lift from his hot waters grams of essentially monotypic bacterial protoplasm!

Since by definition many organisms cannot grow in the extreme environment, we can ask two sets of questions: (1) how does the environmental extreme affect organisms that cannot tolerate it? (2) How is it possible for organisms which *are* adapted to overcome the effects of the extreme factor? One point which we must keep in mind, however, is the other environmental factors of the extreme environment, which must be adequate for the support of life. For instance, the bottom of the ocean floor not only has a high hydrostatic pressure but is also dark and low in organic nutrient concentration. The organisms that we find there will thus be those that are adapted to all the relevant factors, of which high hydrostatic pressure is only one. For this reason it is desirable to study habitats in which a gradient exists for the factor of interest, from extreme to normal, with all other factors remaining the same.

Environmental Extremes

The most commonly considered environmental extremes are for the factors temperature, redox potential (Eh), pH, salinity, hydrostatic pressure, water activity, nutrient concentration, and electromagnetic and ionizing radiations. A brief general review of these factors has been given by Vallentyne (1963). For virtually all of these factors, it is found that organisms with simple structures can grow under more extreme conditions than organisms with more complicated structures, hence the preeminence of microorganisms in extreme environments. There are three ways in which an organism can adapt to an environmental extreme: (1) it can develop a mechanism for excluding the factor from its structure; (2) it can develop a mechanism for detoxifying the factor; (3) it can learn to live with the factor. In some cases, it is the latter situation that obtains, and in fact it sometimes occurs that evolutionary adaptation is such that the organism actually becomes dependent on the factor which for other organisms is lethal. In this chapter we will take some of the environmental factors listed above in turn, and consider the natural habitats where the factor is relevant, and the biochemical mechanisms involved in adapting to it. It should be emphasized, however, that an environmental factor might not always affect an organism directly, but might do so indirectly through its effects on other environmental factors. High temperature, for instance, reduces greatly the solubility of O_2, reduces the viscosity of water, and increases its ionization. We must be certain, therefore, that the effects we are measuring are not due to such indirect effects of the environmental variable.

Temperature as an Environmental Extreme

Temperature is one of the most important environmental factors controlling the activities and evolution of organisms, and is one of the easiest variables to measure. Not all temperatures are equally suitable for the growth and reproduction of living organisms, and it is apt, therefore, to consider which thermal environments are most fit for living organisms. For such a study, high-temperature environments are of especial interest, in that they reveal the extremes to which evolution has been pushed. The high-temperature environments most useful for study are those associated with volcanic activity, such as hot springs, since these natural habitats have probably existed throughout most of the time in which organisms have been evolving on earth.

Although the words *high temperature* will often be used in this book without qualification, the viewpoint of the observer or the group of organisms under discussion will often determine if a given temperature is to be considered high. Thus, a temperature of 50°C would be considered critically high when referring to a multicellular animal or plant but would be considered moderate or even rather low if certain thermophilic bacteria were under discussion. Where necessary, I will qualify the word *high* by appending actual numbers. Another point which should be emphasized at the outset is that one must distinguish between temperatures an organism can tolerate and those at which it can carry out its whole life processes. Thus certain invertebrates can survive exposure to temperatures approaching boiling, although they cannot grow at temperatures above 50°C (Carlisle, 1968; Hinton, 1960). No animal has been found that can carry out its complete life cycle at a temperature over 50°C. In terms of evolutionary success in high temperature environments only organisms able to carry out their complete life cycles are of interest.

Although there may be some doubt that the cytoplasm of an organism in high salt or low pH is subject to the environmental extreme, there is no doubt that microorganisms are isothermal with their environments. A thermophile cannot be a biological submarine with an air-conditioning system, since there is no way for a heat pump to operate in an isothermal environment. (On the other hand, it is possible for organisms to be warmer than their environments if they contain their own internal heating systems, driven by metabolic energy; witness the whale and the submarine.) Thus thermophiles cannot survive at high temperatures because of a thick layer of insulation. Because all of this book deals with high temperature, further discussion of temperature extremes will be reserved for later chapters.

Low pH

"One man's poison is another man's meat" well characterizes the situation for those organisms that flourish in acid environments. In habitats where other organisms rapidly die, these organisms thrive, often multiplying to high density. And the diversity of organisms found in these acid environments is surprisingly high, including not only bacteria, but also algae, protozoa, and invertebrates. Upon understanding the environment, this biological diversity seems more reasonable, for environments made acidic with sulfuric acid are quite widespread on earth, providing many opportunities for evolutionary adaptation.

Acidity is more common in nature than alkalinity, because acidity develops in oxidizing environments, and the biosphere is predominantly aerobic. Sulfuric acid arises from the oxidation of sulfides such as hydrogen sulfide (H_2S) and pyrite (FeS_2) (Dost, 1973). Sulfides are very common in volcanic and other geothermal habitats, in bogs and swamps, and in the sea. In the absence of oxygen, sulfides are stable, but if conditions change and air enters, rapid oxidation, brought about either spontaneously or by action of special sulfur-oxidizing bacteria, quickly leads to sulfuric acid production. As one example, vast areas of low-lying soils along the south-eastern coast of the United States (South Carolina, Georgia, North Carolina) were at one time flooded by the sea, and are rich in sulfides. When these soils are drained for agriculture, acidity rapidly develops, and the soils become completely unsuitable for plants (Dost, 1973). Other areas where such acid soils occur extensively are in The Netherlands, Africa, and Southeast Asia. In The Netherlands such soils are called "cat clays" (kattekleigronden, in Dutch), the word "katte" meaning in the Dutch vernacular any harmful, mysterious influence or quality. Such completely barren soils can be a blight on the landscape, and in underdeveloped countries where every bit of agricultural land is needed they present a real challenge for the agricultural scientist.

Perhaps the most well-known situation of sulfuric acid intrusion is "acid mine drainage." This is found primarily in coal-mining regions, but occurs to a small extent from copper, lead, zinc, and silver mines. In eastern U.S.A., over 10,000 miles of streams and rivers are affected by acid mine drainage, and are unsuitable for human use. Acid mine drainage develops in those coal-mining regions where the coal and overlying rock are high in sulfides, predominantly pyrite. As long as these sulfides are buried beneath the earth, everything is fine, but when mining occurs, air is introduced, and oxidation of the sulfides to sulfuric acid occurs. As rainwater percolates through the earth, it leaches this acid out of the rocks, and streams and lakes that develop are highly acidic. Frequently they are also rich in iron, rendered soluble by the acid waters, and this iron subsequently precipitates and forms a scum for miles around in areas affected by acid mine drainage.

Another form of acid pollution which has recently become known is the formation of acid rains as a result of air pollution. In many parts of the world, rains are falling that are essentially dilute solutions of sulfuric acid. As a result, lakes and rivers are becoming acidic, with important biological consequences. Acidification of rain occurs because of sulfur dioxide pollution of the atmosphere. When sulfur-rich coal or oil is burned to make electricity, the sulfur is oxidized to sulfur dioxide, which spews out into the atmosphere unless special controls are instituted. In clouds or moist air this sulfur dioxide oxidizes to sulfuric acid, which dissolves in the moisture and makes it acidic. Acid rains have been especially noted in Scandinavia and New England, but occur wherever excessive sulfur dioxide pollution occurs.

Seawater is slightly alkaline, witn a pH of 8, and many lakes and river waters are just about neutral, with a pH of 7. Soft water lakes are usually slightly acidic, pH 5 or 6, and many useful agricultural soils also have pH values in this area. Tomato juice has a pH around 4, and vinegar around 3; lemon juice is even more acidic and has a pH around 2. Stomach fluids, which are quite acidic, have pH values between 1 and 2. (However, the acidity of fruit juices is not due to sulfuric acid, but to organic acids such as citric acid, acetic acid, and lactic acid, and the acid of stomach juices is hydrochloric acid.)

The most dramatic development of sulfuric acid environments occurs in geothermal areas. Volcanic gases, coming from deep within the earth, are often rich in hydrogen sulfide, and as this gas reaches the surface of the earth it meets with the oxygen of the air and oxidizes, first to elemental sulfur, and subsequently to sulfuric acid. The classic habitat of this type is Solfatara (the Forum Vulcani of the Romans), a small steaming volcanic crater along the Bay of Naples near Pozzuoli, familiar to tourists for hundreds of years and popular today as a camping site. In Italian, solfatara means "sulfur mine," so-called because sufficient crystalline elemental sulfur was present so that at one time it was mined, and the name solfatara has subsequently been given to the many other sulfur-rich acid geothermal areas around the world. There are many solfataras in Yellowstone National Park, most notably at Roaring Mountain and the Norris Geyser Basin. Solfataras are most easily recognized by the crumbling and bleached nature of the rocks. Sulfuric acid corrosively attacks the rock fabric and causes the rocks to disintegrate into crumbling bits. Elements such as iron, which give rocks their normal color, are leached out, leaving behind a whitish residue containing predominantly quartz, the only mineral stable in very acid conditions. Often the rocks are so destroyed by acid that walking in solfataras is unsafe; beneath a thin surface crust the earth is hollow and hot, and one can easily thrust a foot through into a steaming pool of hot sulfuric acid.

Yet in all these diverse kinds of acidic environments organisms not only live, but flourish. Some, in fact, will live nowhere else. (See Chapters 5, 6, 9 and 12)

Sulfuric acid environments may be as acidic as any environment on earth. Values in solfatara soils are usually pH 2 or below, and acid mine drainage usually has a pH between 2 and 3, but some volcanic crater lakes are even more acidic, with values less than 1. I have even had water samples from small steaming pools which have had pH values as low as 0.2. In an area where sulfuric acid leaches into a hot soil, the evaporation of water may lead to an unusual concentration of the sulfuric acid that is left behind, and we once measured a pH in a Yellowstone solfatara soil of 0.05; even at this low pH we found a living organism, the alga *Cyanidium caldarium*.

Saline Environments

Aqueous habitats exist with total ion concentration varying from essentially zero up to saturation. As ion concentration of the water goes up, some ions precipitate before others. The most soluble are the cations Na^+, K^+, and Mg^{2+} and the anions Cl^-, SO_4^{2+}, and HCO_3^- (or CO_3^{2-} depending on pH). It is thus not surprising that it is these ions that appear in the largest amounts in sea water and the saline lakes. Species diversity in sea water is high and the oceans can hardly be considered extreme environments. Hypersaline waters are devoid of fish and low in diversity of invertebrates; they are clearly extreme.

The precise ion composition of a saline lake is determined by the nature of the rocks surrounding the lake basin. Great Salt Lake, Utah, and many smaller lakes of the U.S. Great Basin, have essentially the ion proportions of sea water in more concentrated form (Livingstone, 1963); such lakes are sometimes called thalassohaline. The Dead Sea, Palestine, is quite different from Great Salt Lake in that it is higher in Mg^{2+} than Na^{+}, and is low in SO_4^{2-}. Lakes high in bicarbonate-carbonate are often called alkali lakes because they are of high pH, values over 10 being reported. One lake in British Columbia is almost pure $NaHCO_3$. Still other lakes are low in chloride and high in sulphate; one lake in British Columbia is composed mostly of magnesium sulfate (Livingstone, 1963). It should also be noted that most of these lakes differ greatly in concentrations of some of the minor but biologically important ions, such as F^-, Br^-, Fe^{3+}, Hg^+, etc.

It must be emphasized, however, that few workers have established that growth of the organisms was taking place at the salinities at which they were found. The mere presence of an organism in a water body does not indicate that it has been grown there; it may have drifted in from fresh water sources. Furthermore, even if the organism can be shown to be alive and growing, the salinity measurements must be made precisely at the site and time at which it is found. Saline lakes vary in salinity considerably throughout the year, due to variations in freshwater drainage. Great Salt Lake, for instance, has a lower salinity in winter than in summer, due to entry of freshwater from snow melt in the surrounding mountains. Also, freshwater is less dense than salt water, and hence will float on top. Organisms developing in this freshwater layer might be mistaken for halophiles.

Even if reproduction is taking place, this does not imply that the organism is optimally adapted to that environment. Only in the case of the halophilic bacteria is the situation reasonably clear; many of these isolates grow optimally in culture in media of about 25% (w/v) NaCl, and show a minimum requirement for growth of 12–20% NaCl (Larsen, 1967). They are thus true halophiles, and even though experiments have apparently never been performed to show that these organisms grow in nature best at high salt concentrations, we would be very surprised indeed if we did not find that to be so. In salt pans, where solar salt is being made, the water at the time its salinity reaches close to saturation often acquires a brilliant pink cast due to the growth of halobacteria, whereas in the more dilute waters, before evaporation is completed, these organisms are not seen. It seems very unlikely that the appearance of these organisms could be due to their mere concentration from diluter waters, and therefore they must be grow-

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