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MORTIMER P. STARR, *Editor*

JOHN L. INGRAHAM,

SIDNEY RAFFEL,

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MORTIMER P. STARR, *Editor*
University of California, Davis

JOHN L. INGRAHAM, *Associate Editor*
University of California, Davis

SIDNEY RAFFEL, *Associate Editor*
Stanford University

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# EVOLUTION OF A MICROBIAL ECOLOGIST

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*Robert E. Hungate*

Department of Bacteriology, University of California, Davis, California 95616

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## EARLY DAYS

A chance to review and comment on career interests is rare, and this opportunity extended by the editors of the *Annual Review of Microbiology* is much appreciated, the more so because I shared an office at Stanford with J. Murray Luck during the period in which he initiated the *Annual Review of Biochemistry.* The title discloses what now appears to be the theme of my scientific life inquiries. The contents are an attempt to understand the forces and factors evoking my ecological interests.

My father, graduating from Washington Agricultural College at Pullman (now WSU), had been profoundly influenced by C. V. Piper, his brother-in-



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law, a professor of biology during the early years of the college, and a dynamic teacher and investigator. Piper's interests extended to all phases of biology, and my father continued this tradition. He worked with the Biological Survey team headed by Robert P. Taylor in the Mount Rainier and Stehekin areas of Washington and developed an extensive study collection of plants, animals, and birds to be used during 46 years of teaching at the State Normal School at Cheney (now EWSU). He had earlier spent a year at Cornell, receiving an M.S. under G. W. Herrick.

I learned much about the plant and animal life of the Cheney area through many outings in the pine woods of the "scabrock" country of Eastern Washington where the loess soil blown in from eruptions in the Cascades had been washed away by floods of water released during the successive ice ages to expose channels on the underlying lava. Courses in invertebrate zoology, mammalogy, ornithology, and entomology at Cheney Normal extended my interests, and I took also as much chemistry and math as was offered. But the institution at that time was basically a 2-year college for training elementary teachers, so when an opportunity arose in 1924 to serve as principal of the three-room school on the Spokane Indian Reservation at Wellpinit, I took the post and taught the 7th through 10th grades for a year before moving to the public schools of Sprague, Wash., for 2 years.

With summer work on the US Blister Rust service at Priest Lake, Idaho, in 1926, including 34 days fighting forest fires, and on the Little North Fork of the Coeur d'Alene in 1927, I had saved $2500 (a good sized sum in those days) and decided to continue my education.

## STANFORD

Stanford University had a famous department of education at that time, and my acquaintances, being teachers, recommended it. Through the catalogue I found that Stanford offered a degree in biology, and I enrolled as a junior, with the intention of qualifying to teach biology in high school. Courses for the first quarter were American Constitution and Ideals under E. E. Robinson (a superb lecturer), general botany with J. McMurphy, and a course in ecology under A. G. Vestal, who had studied with V. E. Shelford at Illinois and who returned to a post there at the end of the year. Acquaintance with fellow students, and an acquired distaste for education courses emphasizing methods of teaching rather than the subject matter, led me after one quarter to abandon the goal of high school teaching. L. L. Burlingame recommended course in physics, chemistry, and mathematics, and I embarked on a basic program in biology, taking T. D. Stewart's organic chemistry in a summer session at the University of California at Berkeley.

The assignment for the ecology course with Vestal was to ride my bicycle out to Jasper Ridge to collect data on soil and air temperature maxima and minima, rainfall, and evaporation at grassland, chaparral, and forest sites. Vestal was sincere and likable and propounded a number of explanations for various observed differences in the collected data. I proposed that we set up experimental plots to test the hypotheses but was stopped by what was then a widespread viewpoint that experiment had no place in ecology. Later, in the summer of 1929, working again for the Blister Rust Service, with Frank Patty in charge of a four-man team to study the ecology of *Ribes* in the Sierras, it was possible to set up field experiments to examine factors affecting seed sprouting. This I liked, though there was no opportunity to observe the results of the experiments. In 1930, with George Draper, I experimented at Maumee, Ohio, on methods for chemical eradication of barberry where sprays could not be used, and showed the effectiveness of transpiration in drawing herbicides into cut ends of branches and down into the crown, killing the plant from within.

That fall Carl Wolfe and Waldo Furgason, who had taken Vestal's place in teaching general biology at Stanford (required of all freshman students), gained their doctorates and took positions. Arthur C. Giese and I were appointed by Burlingame as instructors for the course. We handled over 400 students in each of four classes during the year, also in the lab (3). My association and friendship with Art Giese and his wife Raina in that teaching effort marked one of the most enjoyable and rewarding periods of my life. Art had worked with W. C. Allee, ecologist at the University of Chicago, and had broad interests; he also had been inspired by S. C. Brooks at Berkeley. Art constructed a UV-visible monochromator with which he initiated the precise studies on biological effects of radiation that characterized his entire fruitful career. During this period I married Alice Wolcott, a student at Stanford. She was an immediate help in finishing and typing the Ph.D. thesis, and over the years she has greatly assisted my professional efforts and, with the family, has greatly enriched my life.

While working with Vestal I had been assigned a desk shared with Lewis Thayer and Donald Johansen, adjacent to the office shared by emeritus professor D. H. Campbell with G. J. Peirce, who had studied with Pfeffer. It was a privilege and an inspiration to become acquainted with these sages of botany in America. Also David Starr Jordan was still giving his Thursday evening lectures; one of my prized items is a photograph with him when he came out for a picture with the Zoology Club in the spring of 1928. G. M. Smith and L. R. Abrams, E. MacGinitie and H. Heath (biology), C. F. Luther in mathematics, and G. S. Parks in chemistry were among other professors I enjoyed. D. L. Webster taught a very stimulating course in modern physics and made a lasting impression, not only in the course, but

also in his unmuted discussions with Hanson and other students at the old stand-up counter in the Union Building.

The intructorship in biology provided financial independence but consumed much time in preparation and actual teaching. Art Giese and I arranged that each of us would have free time in one of the quarters in which only one biology course was taught. This enabled us to get on with the Ph.D. I had been taking courses but had selected no program of research. When I decided to take courses at Hopkins Marine Station in the spring of 1931, Lewis Thayer, who had been working at Pacific Grove on possible petroleum genesis from diatoms, urged that I take C. B. van Niel's course. This I did, as the first student, and the only one that quarter. The first lecture was on yeasts, direct examination and count, culture, and fermentation, formally delivered. But that seemed too formal for one student, so thereafter we sat at a table, with a large scratch pad serving as a blackboard for instruction on the coliforms, lactics, sporeformers, and cellulolytics, as well as methyl glyoxal, acetaldehyde, and pyruvic acid, the then current darlings of biochemistry. I thoroughly enjoyed van Niel. He had to be experienced personally for one to understand his contribution. Modern perusal of the notes from that first course do not reveal the spell of his personality, the wisdom of his views, or the great stimulation he imparted to his many students. His was the most profound influence on my scientific career.

## *Termites*

In the Stanford biology course we had an exercise on termites and their cellulose-digesting protozoa as an example of symbiosis. In view of the wide-spread capacity of bacteria to digest cellulose, it seemed to van Niel and me that bacteria might play an important role, and I selected this problem for thesis research. *Zootermopsis angusticollis* and *Zootermopsis nevadensis,* abounding in fallen *Pinus radiata* on the Monterey peninsula, provided copious material.

The experimental demonstration by L. R. Cleveland that the protozoa were essential was already classic, but the inference of cellulose digestion did not seem to be the only possible explanation for the mutualistic relationship between the protozoa and the termite. But failure of all of my series of attempts to demonstrate abundant cellulolytic bacteria (14) with, in retrospect, methods that were probably inadequate, led to the conclusion that the protozoa really were responsible for most of the cellulose digestion. Microscopic examination disclosed that their mass obviously exceeded that of the bacteria. Furthermore, the acid pH in the hind-gut was regarded as unfavorable to cellulolytic bacteria.

At this point a brief but very important conference with Kees van Niel indelibly impressed me. Our questioning of cellulose digestion by the ter-

mite protozoa was not supportable. What course should now be followed? I could undertake some aspect of photosynthesis; Kees' classic studies on bacterial photosynthesis were in full swing at the time, but his advice was to continue the termite research if I felt there was anything more to be done. By that time I had become aware of many facets of termite biology, had been trying to rear them to discover their source of nitrogen, and had attempted in vitro culture of the protozoa. So it was decided to keep on with the termites, and a thesis was finished. It was not a profound contribution, but it covered four years of study on many aspects of termite biology and served as a base for the more definitive studies made later, at Texas until 1940 and again at Hopkins Marine Station on a summer visit in 1937.

## *The Cohesive Force of Water*

Actually, my first published paper (13) was not on termites at all; I had taken a course in plant physiology with Peirce, including the Dixon concept of the cohesion of water as the force responsible for its conduction from soil to the top of tall trees. This was later questioned by a fellow student who was completing experiments interpreted as refuting Dixon's hypothesis. I had in turn questioned the experimental U-tube design used for his water conduit and had suggested that the cohesion of water could be demonstrated if an inverted U-tube were employed. When van Niel, who had been appointed a member of his thesis committee, mentioned concern over the thesis, I voiced my critique of the design and was encouraged to do the experiment that would demonstrate the cohesive force of water.

This was done by drawing out glass tubing in a flame and joining additional lengths as needed to construct a tube 45 feet high, which extended in the stairwell of Jordan Hall from the basement to the third floor and back down again, with both open bottom ends being sealed into the neck of round-bottom 100-ml Pyrex flasks, with side arms joined to the valve stem from an automobile inner tube for applying pressure. Chromic acid cleaning solution was placed in one flask and pressure was applied with a tire pump to force it up 45 feet and down, completely filling the inverted U-tube. Heat was applied with Art Giese's assistance; he poured ethanol down from the top, I lighted it at the bottom, and the flame spread rapidly the length of both tubes, heating the chromic acid solution to thoroughly clean the inner surface of the tube and oxidize impurities on the surface of the glass that would decrease its adhesion to water and constitute a nucleus for formation of a gas bubble. The chromic acid was replaced and the tube was rinsed and filled with boiled cooled water. When the pressure supporting the column of water was removed no gas phase appeared at the top, and water siphoned up and over the 45-foot height, dripping from the slightly lower open end of one arm of the inverted U-tube. Subsequently, the very simple and elegant experiments of Briggs showed a cohesive force of water of about 750

atm, theoretically sufficient to pull a "wire" column of water to a height of 7.5 km!

This may seem nonpertinent to problems in microbiology, but a bacterium exposed to dry air must rapidly develop a negative tension in the water within the cell. According to a hasty calculation, a microbial cell wall capable of withstanding an internal pull up to 7.5 mg/$\mu m^2$ could be filled with water at relative humidities above 38%.

## TEXAS

The termite work was continued at Austin, where I received an appointment in the Zoology Department to teach a year course in general biology for a select group of high school graduates known as Plan II students. A unique feature of the course was use of fresh material. Small transparent aquatic invertebrates were examined under the microscope to see the anatomy and physiology of coelenterates, flatworms, annelids, arthropods, and mollusks. The ciliated nephrostome of *Dero* or *Aulophora,* beating in the coelomic cavity, always aroused my enthusiasm (unfortunately too often not shared by the students), as did their circulatory system and that of *Hyalella,* a crustacean. The students must have been intrigued by some parts of the course; more than half of the first class of 50 went ahead to major in some aspect of biology.

The termite work was continued. Cleveland's postulate of glucose as the end product of the cellulose metabolism of the protozoa failed to account for their energy needs. His observation that pressures of $O_2$ above 1 atm killed the protozoa was consistent with the postulate that they were anaerobic, and my in vitro tests showed this was indeed the case. Warburg respirometric experiments (16) demonstrated that the protozoa not only digested the cellulose, but also fermented the formed sugars to $CO_2$, $H_2$, and acids, of which the most abundant was acetic.

This formed the basis for formulating the termite-protozoa mutualistic relationship as one in which fermentation of the digestible components of wood accomplished the work involved in the maintenance and synthesis of the protozoa, while at the same time providing waste acids that were readily absorbed by the termite and oxidized to maintain and synthesize termites. Experiments on termites obtained directly from nature (15) showed that they, too, produced fermentation gas, explaining Cook's earlier observation of an unidentified gas given off from faunated but not defaunated individuals.

My in vitro measurements on the protozoa identified the gas as $H_2$. The finding that a fermentation product was not used by the termite provided a means for estimating the magnitude of the gut fermentation in intact

termites (21). The amounts of fermentation acid, $H_2$, and $CO_2$ formed by the protozoa (removed directly from the gut) were measured and their ratio was calculated. The $H_2$ produced in worker termites from natural colonies was measured, and the ratio was used to calculate the amount of acid formed. The $O_2$ needed to oxidize that amount of acid (assumed to be acetic) was calculated and compared to the actual oxygen consumption. For one colony of termites the calculated and observed amounts were the same. Protozoa from this colony produced acetic acid, $CO_2$, and $H_2$ in the molar proportion of 1 : 1 : 2, respectively, but for the other colony the $H_2$ was less, a phenomenon unexplained at the time but understandable on the basis of the recent demonstrations by Breznak that $CH_4$ also can be produced.

Success with this quantitative analysis of the termite-protozoa relationship stimulated formulation of the concept that a complete analysis of an ecosystem involved not only identification of species and their activities, but also the amount of the activities. The goal of a complete quantitative analysis of an ecosytem became a dominant element in my thinking and led to later studies of cattle and sheep at Pullman, Wash., (4, 5) and Davis, Calif. (48). The total activity (metabolism) of the ecosystem should be measured, and the precision and validity of the ecological analysis should be tested by the algebraic addition of the individual activities and comparison of the sum with the measured activity of the total system. Adherence to this goal will do much to prevent over-inflation of the ecologist's ego concerning his ecological accomplishments!

The nitrogen nutrition of *Zootermopsis* was also intriguing. Cleveland had observed increases in the number of individuals in small colonies started with ca 10 to 20 individuals fed filter paper as the sole organic food, and concluded that $N_2$ was fixed. My culture attempts (18) gave similar results, but analysis of the initial and final nitrogen content provided no evidence of overall fixation and in many cases the termite nitrogen content decreased. This was true even when cannibalism was eliminated by starting the colony with a pair of alates (young king and queen). A half dozen or so young might be produced, but the total termite nitrogen was still less than that of the original pair, and estimates of the nitrogen in the termites, the unconsumed wood, and the pellets gave no indication of nitrogen fixation or of loss of nitrogen. Later experiments with small colonies of 21 *Kalotermes* showed a small increase in termite nitrogen but no evidence of $N_2$ fixation, and addition of yeast extract or ammonium sulfate somewhat increased the termite nitrogen content.

This led to the initiation of cultures (23), each containing some soil and a large block of wood, and started with a pair of alates, an important experimental refinement eliminating cannibalism and simulating the initiation of natural colonies. The initial nitrogen in the culture was calculated

from analyses of wood (0.045%), soil (0.096%), and alates (5.5%) similar to those used for the cultures. After 5 years, one culture consisted of the original queen, 144 alates, 127 nymphs ready to molt into alates, and 180 other nymphs. Termite nitrogen had increased over 500 times. The fecal pellets were easily separated from the uneaten wood, and analysis of the soil (0.06%), wood (0.058%), and pellets (0.256%) showed that considerable amounts of the nitrogen in the soil and wood had moved into the vicinity of the termite burrows, and that at least one third of the consumed nitrogen had been assimilated into termites. No measurable nitrogen had been fixed. Much of the uneaten wood had decreased markedly in specific gravity due to fungal attack, and presumably fungal filaments, gathering nitrogen from soil and wood, transferred it into the vicinity of the burrows where wood was consumed. The ratio of weight loss of wood (1429 g) to total original nitrogen was about 480.

This remarkable nitrogen economy suggested a great efficiency in its utilization by wood-destroying fungi. This was tested (17) during a vacation in Idaho, where analyses of sound and decayed wood showed a ratio of about 700 to 800 parts by weight of wood decomposed to total nitrogen available, with none supplied from soil and with no evidence of nitrogen fixation. It is tempting to suggest that transfer of protoplasm through the clamp connections to the point of fungal growth may be a factor in this great efficiency, emptying nitrogen from regions where growth has already occurred, and depositing it at the growing tips of mycelial filaments.

Further experiments on termite cultures included the finding of wood-eating *Amitermes,* in which the wood was digested by bacteria instead of protozoa, but in the meantime my interests had turned toward cellulose digestion in cattle.

## *Cellulose Digestion by Cattle Protozoa and Bacteria*

The role of rumen protozoa had aroused much interest ever since their discovery, a role still not completely elucidated. Observations that plant cell walls were ingested had stimulated speculation that the protozoa digested cellulose. The proof that this occurred in termite flagellates made me believe that it might also characterize the rumen ciliates, and I initiated attempts to grow them in vitro, aided by the assumption that they were anaerobic. Initial attempts at culture, using dried grass and powdered cellulose as substrates, were almost immediately successful, and with micromethods a cellulase activity within several species of entodiniomorphs could be demonstrated (19, 20), though for *Entodinium caudatum* no definitive evidence of a cellulase was obtained. Clone strains of the protozoa were carried successfully for almost 2 years with 1- or 2-day 2X dilutions. This was the first successful maintenance of an "artificial rumen."

Although many species of rumen protozoa digested cellulose, it seemed highly probable that it was digested also by bacteria. Their culture had been attempted by others without success. One possible explanation was that some factor peculiar to the bovine was required. The cellulose added to protozoa cultures was in part ingested, but the remainder settled on the bottom of the culture flask and then rose to the surface with the grass particles as fermentation gas formed, and was completely digested after 2 days. Since few protozoa occurred in the surface material, this digestion showed that bacteria were involved, and also that no organic nutrients from the ruminant were required.

The fact that the bacteria grew in the in vitro protozoal culture supported the assumption that they would grow also in pure culture if the nutrients in the protozoal culture were supplied. Shake tubes of agar media containing sterilized protozoa culture and finely powdered cellulose were prepared, inoculated with several dilutions of the protozoal culture, and incubated. After several weeks I observed a colony in a spherical clearing of cellulose in one of the higher dilutions (22). Subculture to a second cellulose agar series was successful, and the cellulolytic capacity was retained after two serial passages through media containing sugar instead of the cellulose. This disproved the then current belief that growth of anaerobic cellulolytic bacteria on sugar caused an immediate loss of their capacity to digest cellulose. But the isolated *Clostridium cellobioparum* occurred in numbers too small for it to be important in the rumen, and further studies were undertaken.

## *The "Hungate" Method*

Difficulty in seeing the cellulose clearings in shake tubes led to use of roll tubes to obtain thin layers in which cellulose digestion and the concerned colony could more easily be seen. It also facilitated detection of differences in the shapes, sizes, and color of bacterial colonies of different types. A binocular dissecting microscope was useful in differentiating desired from contaminating colonies. Since the rumen atmosphere contained 70% $CO_2$, a $CO_2$ atmosphere and 0.5% $NaHCO_3$ was selected as the chief buffer. This necessitated flushing of culture tubes with $O_2$-free $CO_2$ and closure of the tubes with black rubber stoppers, which fortunately were relatively impermeable to gases. The method was well suited also to use of gases as substrates. A balanced salt solution constituted one third of the medium, rumen fluid composed another third, and the last third was a suspension of HCl-treated absorbent cotton, or later, of Whatman #1 filter paper, wet ground overnight in a pebble mill to obtain a suspension so finely divided that each bacterium would be as close as possible to a cellulose particle, enabling a single cell to initiate a colony.

At the time the method was put together I felt that the ecological principles on which it was based were sound, but wide adoption was delayed, first, because the written description of the procedure gave an impression it was unduly cumbersome, second, because the red rubber stoppers used in British Commonwealth countries were quite permeable to $O_2$ (50), and third, because most bacteriologists were wedded to the Petri dish. So far as I am aware A. Kistner in South Africa and Paul Smith at Virginia Polytechnic Institute were the only early investigators who successfully adapted the procedure from the written description. Otherwise, its spread depended on contact with a laboratory in which the relative ease and success of the technique could be observed directly. Students and associates who worked in my laboratory carried the method into other laboratories and so on, until after 25 years it was fairly widely used, modified, and improved as a means for studying organisms susceptible to injury by $O_2$, and requiring its almost complete absence in order to grow.

Success with use of protozoal culture fluid to nourish cellulolytic bacteria led to the obvious extension that rumen fluid should be part of the medium, but not all of it, because it contained bacterial waste products as well as the nutrients. The chosen one third was fortunate, as subsequently found by others.

The first isolated type of cellulolytic rumen anaerobe was *Bacteroides succinogenes* (27, 28), followed soon after by the "yellow" and "colorless" cocci. Also during this period the cellulolytic *Micromonospora propionici* (26) and some sporeforming anaerobes were isolated from *Amitermes*.

A little-noted aspect of the success of isolating rumen bacteria was its dependence on direct isolation in agar dilution tubes, omitting any preliminary liquid enrichment. This is of very broad and fundamental importance. A good enrichment medium should elicit growth from a single bacterium, and this can occur in agar as well as in liquid medium. The separation of individual cells by the agar can even increase chances for growth of a greater variety of cell types.

## *How to Make Do*

Today when millions are spent on research programs it seems inconceivable that the total extramural research budget for these projects at Austin was $200 granted 1 year by AAAS and $400 in another year by Sigma Xi. Fortunately, Royce Skow had shown me how to make a glass electrode pH meter, and at Stanford I had taken a 1-unit chemistry course in glass blowing and had subsequently continued practice in this art. It enabled me to construct Warburg vessels with two sidearms (including the ground glass joints and stoppers), a special bulb of palladium black for absorbing $H_2$, and whatever other chemical glassware was required. A Warburg shaker was