Meetings

Biological Nitrogen Fixation

Now that workers in several laboratories have been able to prepare relatively stable, highly purified proteins catalyzing the reduction of nitrogen to ammonia, and in view of the fact that simple, sensitive assays are available, the elucidation of the mechanism of biological nitrogen fixation in free-living microorganisms and symbiotic tissues appears imminent. This was revealed at a colloquium on biological nitrogen fixation, held at Sanibel Island, Florida, 1 to 3 December 1966, under the sponsorship of the National Science Foundation and the Graduate School and Institute of Food and Agricultural Sciences of the University of Florida.

Several laboratories reported success in the purification of catalytic proteins comprising "nitrogenase," the enzyme complex catalyzing $N_2 \rightarrow 2NH_3$. An iron-molybdenum protein and an iron protein from *Azotobacter vinelandii*, required for nitrogen reduction, for H_2 evolution dependent on adenosine triphosphate (ATP) and for the coincident release of inorganic phosphate (Pi), were prepared by high-speed centrifugation and polyacrylamide-gel column chromatography. While discussing criteria of purity of these preparations, W. A. Bulen (C. F. Kettering Research Laboratories) presented ultracentrifuge data which demonstrated the separation of the major components of the P-200 fraction into at least two components after oxidation by O_2. *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), a buffer superior to the inhibitory phosphate or potentially hazardous cacodylate, gave minimal inhibition with increasing concentration and a broad *p*H activity curve with the optimum at *p*H 7.25 at a buffer concentration of 50 m*M*. The ratios of Pi to 2e averaged 4.88 over a wide range of *p*H and nitrogen pressures.

Because the proteins comprising the nitrogen-fixing systems of *Clostridium pasteurianum* are involved in oxidation-reduction reactions and contain specific metals, the names of molybdoferredoxin (MoFd) and azoferredoxin (AzoFd) were proposed by L. E. Mortenson (Purdue). Molybdoferredoxin was about 78 percent pure, based on a molybdenum content of 1 atom per molecule and a molecular weight of 100,000; at this stage of purity, it contained 1 atom of Mg and 12 atoms of Fe per atom of Mo. The second component, azoferredoxin, was purified about 30-fold and contained 6 atoms of Fe per molecule at this stage of purity. Both components were inactivated by oxygen. Molybdoferredoxin could be frozen with little loss of activity; in contrast, azoferredoxin lost partial activity under all conditions of storage. Neither protein was present in extracts of cells grown on ammonia, although a small amount of molybdenum was found in an isolated high-molecular-weight component. In addition to these components, Mortenson and Bui suggested that a third protein (molecular weight of about 40,000 to 60,000) was required for ATP-dependent H_2 evolution from $Na_2S_2O_4$, and presumably for N_2 reduction.

R. H. Burris (Wisconsin) reported on clostridial preparations of sufficient purity and stability to permit a more detailed study of the nitrogen-fixing and hydrogen-evolving reactions. These preparations were made by extracting dried cells, removing salts on Sephadex G-25, removing nucleic acids with streptomycin, and precipitating the active protein with cold acetone or (preferably) alcohol at a final concentration of about 12 percent by volume. The resuspended precipitate had a specific activity near 45 and showed a small dilution effect. Acetylene and N_2 depressed H_2 evolution, because N_2 or acetylene reduction compete for electrons with the H_2-evolving systems. The CO-sensitive hydrogenase and ferredoxin were greatly depleted in the solvent-precipitated preparations; addition of ferredoxin enhanced the electron flow to the CO-sensitive hydrogenase. In the system with $Na_2S_2O_4$ as the electron donor and creatine phosphate–creatine phosphokinase for ATP generation, 4 to 4.6 ATP's were required per pair of electrons donated to yield H_2 or to reduce by one-third N_2. A method for rapid (about 5 hours) preparation of extracts from *A. vinelandii* with a purity sufficient for study of the properties of the enzyme complex was also developed. In this case, fresh cells of *A. vinelandii* were disrupted with a French press, centrifuged, dialyzed, heated anaerobically, treated to remove nucleic acids, and centrifuged again.

The opening discussion by P. W. Wilson (Wisconsin) considered problems, investigated in his laboratory, relating to nitrogen fixation, nitrogenase induction, and hydrogen inhibition in some of the less exploited nitrogen fixers. Considerable $^{15}N_2$ incorporation was obtained with *Desulfovibrio desulfuricans* and *Desulfovibrio gigas* but not with *Desulfotomaculum nigrificans*, with stock cultures and fresh isolates from Lake Mendota mud. The pN_2 which induces nitrogenase was investigated in organisms that show a "diauxie" growth curve on limiting amounts of ammonia in an atmosphere of N_2. In *Klebsiella pneumoniae*, the pN_2 for induction must be very low, because it was satisfied by the nitrogen contaminating high-purity helium; in *Azotobacter*, it could not be determined because of the relatively high concentrations of N_2 in the O_2 required by this organism. The nature of the inhibition of N_2 fixation by H_2 in cell-free extracts of *Azotobacter* incubated in the absence of O_2 was again found to be competitive. The K_{N_2} in such preparations was 0.15 atm, rather than the 0.01 to 0.02 atm obtained with growing cells. Similar values were obtained with *K. pneumoniae*.

Some characteristics of the H_2-evolving systems of *A. vinelandii* and their relationship to N_2 fixation were given by R. C. Burns (C. F. Kettering Research Laboratories). Magnesium was replaceable by Mn^{++}, CO^{++}, Fe^{++}, or Ni^{++} in the ATP-dependent H_2 evolution reaction; the divalent

cation : ATP ratio, rather than the divalent cation concentration, was critical. The average corrected ratio of Pi released to H_2 evolved in a system supplemented with $Na_2S_2O_4$ was 4.2. A 1 : 1 stoichiometry for H_2 evolution to $Na_2S_2O_4$ consumption was suggested. Kinetic studies supported the idea that H_2 evolution, Pi release, and N_2 reduction are closely linked phenomena.

R. W. F. Hardy (Central Research Department, E. I. du Pont de Nemours and Co.) reviewed work done in association with P. Knight and E. Jackson on flavodoxin, substrates of nitrogenase, and characteristics of their reductions, HD exchange, and mechanisms of N_2 fixation. Flavodoxin, the electron-transferring protein from *C. pasteurianum* grown on limiting iron, contains 148 amino acids and one flavin, FMN, which is possibly bound through the SH of the one cysteine residue. It has a redox potential of −280 mv and replaces bacterial ferredoxin in nitrogen fixation, as well as other ferredoxin-mediated reactions, with relative efficiencies of 31 to 171 percent.

A detailed study was made of a variety of substrates reduced by partially purified nitrogenase preparations from *A. vinelandii* and *C. pasteurianum*. A comparison was made for 18 different characteristics of the following reactions: (i) $N_2 \rightarrow 2NH_3$; (ii) $N_3^- \rightarrow N_2 + NH_3$; (iii) $N_2O \rightarrow N_2 + H_2O$; (iv) $C_2H_2 \rightarrow C_2H_4$; (v) $HCN \rightarrow CH_4 + NH_3$ and CH_3NH_2; and (vi) $CH_3NC \rightarrow CH_4 + CH_3NH_2$ and $C_2H_6 + C_2H_4$. Similar requirements for ATP generators, $Na_2S_2O_4$, an iron-molybdenum protein fraction and an iron protein fraction, distribution of enzyme activity, and inhibition by CO provided convincing, indirect proof that all reactions were catalyzed by the same enzyme, nitrogenase—that is, that no unique activation of N_2 occurs. The observed K_m's were not identical, but the rate of electron consumption was constant. Thus, the rate of electron activation was suggested to be the limiting reaction for N_2 fixation by cell-free extracts.

Analogs of acetylene and cyanide provide a powerful tool for mapping the active sites of nitrogenase. Reduction of C_2H_2 to C_2H_4 accompanied by gas chromatography and detection by H_2 flame ionization was proposed as an important and extremely sensitive assay for N_2-fixing enzymes. This was borne out by several reports on symbiotic systems.

The work of the Agricultural Research Council's Unit of Nitrogen Fixation (University of Sussex, England) was explained by J. R. Postgate. Cell-free preparations of *Azotobacter chroococcum* with behavior comparable to that of *A. vinelandii* (reported by Bulen) reduced methyl isocyanide—as well as azide, cyanide, and acetylene —relatively rapidly, yielding methane and methylamine plus significant amounts of ethylene and ethane. This unusual reduction pathway could be mimicked chemically if isocyanides were coordinated to platinum. With *A. chroococcum* growing in continuous culture, nitrogen was the growth-limiting component at all dilution rates; N_2 fixation was most efficient in terms of carbon utilized at low pO_2 values, and oxygen could become toxic in conditions of high aeration. A cell-free pellet from an unequivocally N_2-fixing strain of *Desulfovibrio* catalyzed N_2 fixation during ATP-activated hydrogen evolution. Ferredoxin and rubredoxin were partially purified from a related strain of *Desulfovibrio*. An examination of *Azotobacter* species and several putatively N_2-fixing species of *Pseudomonas* and *Nocardia* obtained from culture collections revealed that none fixed N_2. However, *Beijerinckia* and *Derxia* did so, the latter in continuous culture with the unusually high efficiency of 50 to 100 μg of nitrogen per milligram of carbon source. On the chemical side, systems potentially capable of N_2 fixation, including nitrogeno- and hydrido-complexes of ruthenium or titanium, as well as products of the decomposition of diimide in the presence of $^{15}N_2$, were studied. Thus, it appears that chemical studies may now contribute greatly to our knowledge of biological N_2 fixation, and vice versa.

N. E. R. Campbell (University of Manitoba, Winnipeg) reported on the use of the radioisotope $^{13}N_2$, for screening aerobic free-living isolates obtained from subarctic soils. Details of $^{13}N_2$ production by 50-Mev proton bombardment of ^{14}N in melamine were presented, along with evidence of the purity of the product. Despite the short half-life of the isotope (10.05 minutes) and difficulties in quantitative analysis, this assay system was most useful for qualitative studies on whole-cell and cell-free systems. Incorporation of $^{13}N_2$ as low as 1 to 5 $\times$ 10^3 atoms can be significantly resolved with the detection system in use. There are current investigations of nitrogen fixation in cell-free preparations of a species of *Rhodotorula* and a species of *Pullularia* and in a bacterial isolate resembling *Chromobacterium violaceum*.

Although as yet no high-purity cell-free system has been prepared from symbiotic tissues, several reports indicated that this may soon be done. H. J. Evans (Oregon State University, Corvallis) reported that soybean nodules catalyzed the reduction of acetylene to ethylene at approximately 10 μM per hour per gram of fresh nodules. The optimum concentrations of oxygen and acetylene for the reaction were 0.2 and 0.1 atm, respectively.

Radioactivity from labeled propionate was incorporated into the heme moiety of soybean nodule leghemoglobin at rates approximately equal to rates of incorporation of radiocarbon from several intermediates of citric acid cycles. Even though propionate could not be detected in soybean nodules, bacteroid extracts catalyzed the formation of propionate from lactate when reduced nicotinamide-adenine dinucleotide, coenzyme A, ATP, and Mg^{++} were present.

A study of the reasons why cobalt deficiency in *Rhizobium* results in the production of giant cells led to the demonstration of the presence of a B_{12} coenzyme-dependent ribonucleotide reductase in extracts of *R. meliloti*. The enzyme required reduced lipoic acid as an electron donor and catalyzed the reduction of guanosine triphosphate and certain other nucleotides. In view of the fact that cobalt-deficient *R. meliloti* cells are known to be deficient in B_{12} coenzyme, the normal synthesis of DNA probably failed to proceed because the normal function of the ribonucleotide reductase was blocked by cobalt deficiency.

C. Sloger and W. S. Silver (University of Florida) have also applied Hardy's sensitive acetylene reduction method to intact and fragmented nodular tissue of N_2-fixing legumes and nonlegumes in an attempt to analyze the conditions under which fixation occurs in homogenates. In confirmation of results obtained with $^{15}N_2$, reported previously by these workers and independently by F. J. Bergersen, there was an absolute O_2 requirement for

acetylene reduction, even though the system was sensitive to oxidation and had to be protected with an inert gas during homogenization. When the gas mixture was 5 percent acetylene, 10 percent oxygen, and 85 percent helium, ethylene production proceeded linearly for 7 hours in *Myrica cerifera* and 2 hours in *Glycine max*. This parallels the time course of $^{15}N_2$ fixation for these tissues. Ethylene production was roughly equivalent to acetylene disappearance in *Myrica* (5.8 μM as opposed to 5.0 μM per gram of fresh weight per 4 hours). Acetylene reduction, which could only be detected in nodulated roots, paralleled N_2 reduction patterns in other species of leguminous and nonleguminous plants. Acetylene reduction in homogenates supplemented with $Na_2S_2O_4$ and an ATP-generating system was only one-hundredth that of intact tissues on a comparative weight basis, a relationship not unlike that found for $^{15}N_2$ with *Myrica*.

R. V. Klucas (Wisconsin) described the use of a simple modification of the French pressure cell which permits disruption of nodules in the absence of air. Soybean root nodules broken anaerobically and exposed aerobically to $^{15}N_2$ fixed N_2 at reasonable rates.

Work on the primary products of nitrogen fixation in the root nodules of *Serradella* as studied with $^{15}N_2$ was summarized by I. R. Kennedy (University of Western Australia, Nedlands). Although the labeling patterns obtained were qualitatively consistent with the formation of amino acids and amides from ammonia in a single metabolic pool, a kinetic analysis suggested the occurrence of two or more internal ammonia pools in the early reactions of N_2 fixation. After 45 seconds of exposure to $^{15}N_2$, the label was in ammonia, glutamic acid, glutamine, aspartic acid, alanine, and asparagine; the asparagine was least labeled. The ammonia pool was saturated within 5 minutes, whereas amino acid and amide enrichment continued at a linear rate. Pulse labeling and displacement experiments with $^{15}N_2$ indicated that ammonia was a primary intermediate and that glutamic acid and glutamine were the primary amino compounds.

The informal gathering of the colloquium, which was similar to that of previous colloquia [*Science* **147**, 310 (1965); *ibid.* **151**, 1565 (1966)], provided an opportunity for an exchange of current data and ideas between workers interested in the mechanism of biological nitrogen fixation, a timely topic of considerable theoretical and practical importance.

W. S. SILVER
Department of Bacteriology, University of Florida, Gainesville 32601

Retirement Crisis

Experts on aging attended a workshop, 3–5 April 1967, in Washington, D.C., aimed at conceptualization of the retirement crisis in developmental terms. The conference was a first and most important step toward stimulating systematic investigation of retirement as a period in human development. There is reluctance, or at least inertia, in perceiving development as life-long. From a life-history viewpoint, it is known, at least in general terms, where the retiring person "came from" developmentally. As yet unknown is the nature of the transition from the producer-adult stage to the end-of-life leisure stage. "Crisis" is used in the sense of a turning point in development that will markedly affect subsequent development.

The discussion strove to formulate researchable questions and hypotheses that will answer the question, "What is the retirement crisis?" Participants agreed that retirement is an amorphous concept that can be viewed as a process, an event, or a transitional period. Retirement as a homeostatic crisis was discussed from various viewpoints. Donahue (University of Michigan) raised the question, "What is the gestation period of retirement?" The general feeling was that few arrive at retirement without having thought about it. This raised the question of differences in occupations.

Eisdorfer (Duke University) listed the covariants age, health, sex, socioeconomic status, nature of job, and emotional balance as determiners of one's subjective and financial readiness and preparedness for objective retirement.

The group turned to the meaning of work, Eisdorfer pointing out that this is a central issue of the retirement crisis. Medically, nothing is more damaging to the older person than retirement from activity, remarked Bortz (Lankenau Hospital). The concept of work as morally, physically, and financially necessary and good is being challenged, noted Eisdor-