Meeting

Nitrogen Fixation

A group of American scientists with common interests in problems of biological nitrogen fixation participated in an informal colloquium at the University of California's Sagehen Field Station 30 September 1965. The gathering, sponsored by the M. Theodore Kearney Foundation for Fundamental Research in Soil Science, of the University of California, Davis, provided opportunity for the informal exchange of information and stimulated critical discussion and integration of ideas.

P. W. Wilson (University of Wisconsin) opened the sessions with an informal discussion of "some biology of biological nitrogen fixation." Observing that most of the contributions would deal with the biochemistry and enzymology of nitrogen fixation, he outlined several problems which concern the microbiologist. Among these are: stability of the strains most extensively used in current investigations, *Clostridium pasteurianum* W-5 and *Azotobactor vinelandii* O; cell-free fixation by strains other than these, especially by freshly isolated strains; and induction of the nitrogen-fixing system in these organisms.

Although practically all the studies on cell-free fixation have been made with the strict anaerobe *C. pasteurianum* W-5 or with the strict aerobe *A. vinelandii* O, comparative studies with the facultative anaerobes such as *Rhodospirillum rubrum, Klebsiella pneumoniae*, and *Bacillus polymyxa* should not be neglected. Using essentially the system described by Bulen [*Biochem. Biophys. Res. Commun.* **17**, 265 (1964)], workers in the Wisconsin group have obtained consistent cell-free fixation with *K. pneumoniae* M5a1 (specific activity, 4 nanomoles of N_2 per milligram of protein per minute) and *B. polymyxa* strain Hino (specific activity, 7 nanomoles of N_2 per milligram of protein per minute).

L. E. Mortenson (Purdue University) described methods for separating the nitrogen-fixing system of *Clostridium pasteurianum* into three distinct protein components in addition to those required for the production of adenosine triphosphate (ATP) and reduced ferredoxin. The first component contained Mo and Fe in the ratio of 1:3, whereas the second component contained Fe. The third component has not been purified yet. Neither of the first two components was synthesized by cells grown with ammonia as nitrogen source, whereas the third component was present in ammonia-grown cells. All three of these components of the nitrogen-fixing system are required also for the electron-requiring, ATP-utilizing system (or the ATP-requiring evolution of hydrogen from dithionite). These similar requirements suggest that the system that requires electrons for the utilization of ATP is part of the nitrogen-fixing system. The one exception is that CO inhibits nitrogen fixation but not the electron-requiring utilization of ATP (or the ATP-requiring evolution of H_2 from dithionite).

Discovery in extracts of *C. pasteurianum* of an enzyme that transfers electrons from reduced ferredoxin to oxidized nicotinamide adenine dinucleotide was reported. The enzyme requires as a cofactor either acetyl-coenzyme A or some compound in the butyric-acid-synthesizing system.

A mechanism and some molecules of biological N_2-fixation were described by R. W. F. Hardy and E. Knight, Jr. (E. I. du Pont de Nemours and Co.). Nitrogen-fixing extracts of *Clostridium pasteurianum* and *Azotobacter vinelandii* were shown to possess a ferredoxin-independent, CO-insensitive, reductant-dependent adenosine triphosphatase, and those of *C. pasteurianum* were shown to possess the related ferredoxin-independent, CO-insensitive, energy-dependent H_2 evolution. Utilization of ATP for activation of electrons for reduction of N_2 or for activation of protons was proposed as a common nitrogen-fixation mechanism of both the symbiotic and the aerobic and anaerobic asymbiotic systems. The ATP reaction releases phosphate with a maximum ATP/H_2 ratio of 2 for *A. vinelandii*. The isolation and characteristics of two homogeneous proteins, a non-heme iron protein and a flavoprotein (flavodoxin), from nitrogen-fixing extracts of *C. pasteurianum* were described. The enzymically or chemically reduced non-heme iron protein produces an electron-paramagnetic-resonance spectrum at low temperature with the magnetic field of resonance at 1.93, 1.95, and 2.00, has a molecular weight of 24,000, contains an atom of labile sulfide, and may be involved in the electron-transport system of nitrogen-fixation by *C. pasteurianum*. Crystalline flavodoxin obtained from cells grown in a low-iron medium has a molecular weight of 14,600 and an absorption spectrum similar to that of a flavoprotein. It substitutes for ferredoxin in the generation of acetyl phosphate from pyruvate, the evolution of hydrogen from dithionite solutions, in nitrogen-fixation from pyruvate and N_2 by ferredoxin-free extracts of *C. pasteurianum*, and in O_2 evolution by washed, illuminated chloroplasts.

Recent work by R. H. Burris and H. C. Winter (University of Wisconsin) was summarized. Although the capacity to incorporate NH_3 into organic compounds is much diminished in extracts from dried cells compared to that of fresh intact cells of *Clostridium pasteurianum*, J. P. Vandecasteele has shown that added nicotinamide adenine dinucleotide (NAD) enhances the NH_3-incorporation capacity remaining in extracts supplied pyruvate. When $N^{15}H_3$ was supplied, the glutamic acid had the highest N^{15} concentration among the amino acids recovered; however, spectrophotometric tests gave no evidence of glutamic dehydrogenase. Most active incorporation of $N^{15}H_3$ occurred when Mg^{++}, glutamate, and an ATP-generating system were added. These supported glutamine synthesis at a rate approximating the rate of disappearance of NH_3.

By freeing *C. pasteurianum* extracts of dialyzable cofactors by passage through an anaerobic column of Sephadex G-25, T. O. Munson was able to show that N_2 fixation by extracts from *C. pasteurianum* supplied with pyruvate is dependent on ATP, phosphate, Mg^{++}, coenzyme A, and thiamine pyrophosphate, as well as on the non-dialyzable cofactor ferredoxin. When acetyl phosphate and H_2 replaced pyruvate, only ATP, Mg^{++}, and ferredoxin were required as cofactors.

The locus of nitrogen fixation in root nodules is not clearly defined. Bergerson has reported, on the basis of exposure of nodules to $N_2{}^{15}$ for periods of

15 minutes or longer, that fixation occurs on a membrane which surrounds groups of bacteroids in the nodule. R. Klucas has repeated the Bergerson experiments but has never found the N^{15} concentration in the membrane fraction as high as that in the soluble fraction from nodules. When the period of exposure to $N_2{}^{15}$ was shortened to 3 minutes, the highest concentration of N^{15} still was in the soluble fraction. When $Na_2S_2O_4$ was supplied as an electron donor and creatine phosphate-creatine phosphokinase as an ATP-generating system, crushed soybean or cowpea nodules failed to fix N_2. It still is necessary to retain the organization of leguminous nodular tissue to achieve N_2 fixation, as crushing abolishes the capacity.

R. Schöllhorn examined the effect of hydrogenation catalysts on H_2-supported fixation by *C. pasteurianum*, and reduction of inorganic nitrogen compounds. A 5-percent palladium-charcoal catalyst decreased fixation supported by pyruvate but enhanced fixation supported by H_2-acetyl-phosphate. Hydrazine was not reduced by the H_2-acetyl-phosphate system; however, azide was reduced to ammonia. This reduction did not occur in extracts heated to 60°C for 10 minutes.

H. C. Winter examined several symmetrical nitrogen compounds for reduction or competitive inhibition of fixation by *C. pasteurianum* extracts. Diethylazodicarboxylate caused a strong inhibition initially, but as it decomposed the inhibition was relieved. Anthracene-9,10-biimine, which decomposes slowly to diimide, had no effect. Maleic hydrazide and 6-methyl-3-pyridazinone also were without effect at concentrations up to $10^{-3}M$.

J. C. Hwang separated an extract from *C. pasteurianum* on a Sephadex G-100 column and recovered two fractions which had specific activities of 32 and 25 when combined with an extract heated to 60°C and pyruvate.

Crude extracts from *A. vinelandii* fixed N_2 with acetyl-phosphate-H_2 or with creatine phosphate, creatine phosphokinase, and $Na_2S_2O_4$. One sedimented fraction had a specific activity of 7 nanomoles of N_2 fixed per milligram of protein per minute. Preparations solubilized with digitonin or sodium deoxycholate were inactivated. Treatment with streptomycin sulfate (9 mg/ml) removed 39 percent of the protein with no loss of nitrogen-fixing activity.

R. C. Burns (Charles F. Kettering Research Laboratory) described a procedure for the producible preparation, under anaerobic conditions, of cell extracts of *Rhodospirillum rubrum* which catalyze N^2 reduction and ATP-dependent evolution of H_2. Freshly harvested cells were suspended in $0.1M$ phosphate buffer, *p*H 7.5, and ruptured in a French pressure cell or by treatment with high-frequency sound. After being adjusted to *p*H 7.5, the crude extract was centrifuged for 3 hours at $144{,}000g$. The supernatant was passed through a Sephadex G-25 column. The material in the brown band following the void volume caused both N_2 reduction and ATP-dependent H_2 evolution and was relatively free of interfering substances.

H. J. Evans, S. Russell, and G. Johnson (Oregon State University) have continued to study the biochemistry and nutrition of organisms that fix atmospheric nitrogen. With the techniques of Bulen and associates, cell-free nitrogen-fixation experiments were carried out with extracts of *Azotobacter vinelandii*, some of which had been exposed to light or treated with charcoal in order to remove B_{12} coenzymes. These extracts consistently catalyzed the fixation of nitrogen, but no evidence was obtained that B_{12} coenzymes were directly required in the fixation process.

Experiments with the small aquatic fern *Azolla filiculoides* have confirmed that this organism requires cobalt for growth in symbiosis with *Anabaena azollae* in a medium lacking fixed nitrogen. When adequate nitrate nitrogen was supplied to the symbiont, however, it could not be demonstrated that the fern required cobalt, although the number of *Anabaena azollae* cells in fronds of the fern was greatly reduced when inadequate cobalt was supplied.

All attempts to demonstrate nitrogen fixation by extracts of soybean nodules from soybean plant were unsuccessful. The fractionation of nodule extracts revealed the presence of proteins that may be important in legume nodule metabolism. One protein fraction eluted from a diethylaminoethyl-cellulose column apparently contained a flavoprotein able to catalyze the transfer of electrons from reduced NAD (NADH), but not from its phosphate, to 2-6 dichlorophenolindophenol and to benzyl viologen. Another fraction eluted from a DEAE-cellulose column by high concentrations of salt appears green to brown in color, has an absorption shoulder at 410 $m\mu$, and contains labile sulfur and nonheme iron. This protein does not substitute for ferredoxin in enzyme assays requiring this component. The properties and possible physiological roles of the NADH dehydrogenase and the nonheme iron protein are under investigation.

W. S. Silver (University of Florida) reported experiments conducted with C. Sloger on nitrogen fixation by excised root nodules and root-nodule homogenates of the shrub *Myrica cerifera*. The nitrogen-fixing activity of fleshy nodules was several times greater than that of woody nodules. The ability of excised nodules to incorporate $N_2{}^{15}$ declined rapidly under a variety of storage conditions. Homogenates of nodules ground under helium fixed $N_2{}^{15}$ in the presence of $Na_2S_2O_4$ when the gas phase contained 0.2 atm O_2. Although homogenates were active in many experiments (for example, enrichments of 0.274 atom percent excess N^{15} in 4 hours), the number of negative experiments was sufficiently large to suggest that all of the conditions required for fixation are not known.

C. C. Delwiche (University of California) reported upon studies done in collaboration with T. Hartmann. Reports of European workers that seedlings of *Pisum sativium*, when exogenously supplied with vitamin B_{12}, fixed atmospheric nitrogen were explored with the use of isotopic nitrogen. No evidence for nitrogen fixation was found.

Efforts to obtain cell-free preparations from legume nodules capable of fixing nitrogen were unsuccessful. These included various methods of preparation, both aerobic and anaerobic, and a variety of electron donors and acceptors. When nodules, after excision from the plant, were cut into slices approximately 0.5 millimeters thick, they still fixed nitrogen, but at a considerably reduced rate. Dicing the nodules into 0.5-millimeter cubes resulted in still further reduction of fixation ability, and when the nodules were homogenized, capacity for fixation disappeared completely.

Collaborative work with P. J. Zinke and C. M. Johnson was also reported. These studies consisted of an examination of 13 species of *Ceanothus* for the ability to fix nitrogen. Excised nodules from all species tested were able to fix nitrogen at significant rates. It was calculated that a fixation of nitrogen at the rate of 60 kilograms per hectare per year might be expected under field conditions.

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