Grant N· NrG 39-030-004

Title: The molecular biology of nitrogen fixing nodules in common legumes.

Institution: The University City Science Center, Philadelphia, Pa.

Annual Report: July 15, 1969

1. Administration:

The administration of the grant was transferred to the University City Science Center, a non-profit corporation, on 1 August 1968. This is the first annual report under the new administration.

The Science Center accepted this research at an indirect cost figure of 13% of salaries, the figure in effect under the previous grant, NsG 335, with the University of Pennsylvania. The Center is increasing its overhead demand to around 50% following a study of its costs by government auditors.

2. Laboratory facility:

The new facility has been in operation for one year at 249 South 24th Street, Philadelphia, 19103 (215-LO9-2882). Two major installations are still required but are being delayed pending information on funding starting 1 October 1969: the fume hood and exhaust system, and the chromatography columns needed for the sequential amino acid analysis and protein purification.

Dr. Elizabeth Thorogood
249 South 24th Street
Philadelphia, Pa. 19103
1-215-LO9-2882

Scientific investigation:

a. Field work: The following nodule supplies were obtained:

Bur Clover	California	2.8 Kg	6.2 lb.
Lupine, annual	"	1.1 Kg	2.4 lb
Soy A	Illinois	6.8 Kg	15.0 lb
Soy B	"	4.3 Kg	9.5 lb
Soy M	"	2.0 Kg	4.4 lb
Lespedeza	"	0.15 Kg	0.25 lb
Vetch	California	sampling	
Sweet Pea	"	sampling	
Pea	Maine	sampling	

Soy A and B refer to soybean inoculated with pure strain cultures of rhizobia (Urbana Laboratories, Urbana, Illinois). Soy M is soybean inoculated with the commercial mixture of rhizobia.

Whereas in 1968 18 pounds of pea nodules were collected, this year owing to weather and field conditions no collection was attempted. In a sampling it was discovered that 50% of nodules were decomposed after reaching moderate development. A second sampling one month later indicated no improvement.

Measurements of the yield and rate of collecting were made:

Soybean B: In a 3 hour period, 757 soybean B plants were dug, yielding 450 grams of washed nodules and averaging 75 grams nodules per man hour. This is reasonable when the soil is neither muddy nor brick-hard. These 757 plants were dug from a row 78 feet in length so that there were roughly 10 plants per foot, and 5.6 grams obtained per linear foot.

Soybean B, in a larger sampling taken another day, produced 1550 grams washed nodules in 330 linear feet, equivalent to only 4.7 grams per foot.

Thus with our typical farmer's plantings of 10 to 12 plants per linear foot, a yield of around 5 grams per foot can be expected in a good year, with 500 to 600 mg nodules per plant.

Lespedeza nodules are much smaller than soybean. In one measurement, 500 plants were obtained over 3 hours and yielded 11 gramscf washed nodules rather contaminated with root hairs. The collecting is thus approximately 4 grams per hour, with 22 mg nodules per plant.

A second count of lespedeza gave 6 grams nodules per man-hour. Thus an average figure of 5 grams per man-hour is reasonable provided the soil is workable - which is not always the case.

In summary: 2 soybean plants yield 1 gram nodules, whereas 50 lespedeza plants may be required to obtain 1 gram nodules.

Over 26,000 soybean plants were dug for their 13.2 Kg nodules. This means almost 1,000 plants required per pound nodules.
Our 150 grams lespedeza came from 7,500 plants, approximately.

We estimate, by extrapolation, that over 2,600 linear feet of soybean field were dug out this summer for our harvest; roughly half a mile.

The one thing to remember in using these figures is that not all plants dug bear nodules. For reasons of fertility, or flooding, or various other unknowns, a certain portion of plants dug yield nothing. And if conditions are rainy, or muddy, or brick-dry, or if work is conducted through an old road bed, as is required with our lespedeza, the man-hour yield suffers. This season, with good conditions, in our 3-hour collection period we got 2/3 as many lespedeza plants as soy, and 1/45th the nodule weight from them.

Mr. William M. Taylor of the Urbana Laboratories made a second experimental planting of different legumes for our study, and a report on this will be submitted when the data are assembled. The plants are still growing.

b. Extraction of nodules: (Reference should be made to the Semi-annual report of January 15, 1969.)

Pea nodules: Our best technique of extracting the heme protein from the pea nodules is: to use an atmosphere of carbon monoxide combined with hydrogen; to extract in mildly acid buffer;and to dialyze the ammonium sulfate-heme protein paste against Tris buffer, changing to distilled water, first washing the dialysis sac thoroughly. Such an extract of heme protein must be used promptly either in study or extended purification be it by electrophoresis or column chromatography. The paste is unstable frozen, unlike most other plant extracts so far studied; it loses color within weeks. This phenomenon is important, reflecting as it does some extreme of structure in the protein whereby it cannot hold onto the heme.

Pea extracts are exceptionally viscid. The viscous material hampers salt fractionation, and comes down finally, incompletely, between the 24-26% w/w ammonium sulfate cuts. This precipitate can be dialyzed and concentrated to give a thick whitish gel in considerable quantity. The contaminant gives strongly positive Molisch and anthrone tests, weak or negative Benedict's test, and precipitates as a gel with ethanol. It does not hydrolyze noticeably with amylase. It does not stain with iodine.

The occurrence of a viscous polysaccharide-containing material is not unique with pea. It occurs somewhat in bur clover. It is also present in soybean, accumulating in the nodules as the nodules mature on the root. However, in soybean there has never been the technical problem seen in the pea extractions. Aside from the fact the contaminant may differ, the quantity is less, and the soy heme protein is considerably more stable to manipulation.

Lupine: Lupine gives a watery extract, like guar. The ammonium sulfate paste retains the cherry red color of the CO derivative for as long as five months in the freezer at -20C. The procedure developed for pea nodules is applied to lupine also. Lupine heme protein is unstable and decomposes during the extraction procedure used with soybean or lespedeza.

Bur clover extracts are somewhat viscid. Viscosity gives no difficulty. A fatty-like detritus, however, floats on the high specific gravity salt solutions during fractionation and prevents clean cutting. Detritus also clogs the pores of dialysis tubing, precipitating while the salt dialyzes from the tubing and likewise when water is evaporated through the tubing, in our refrigerated technique of concentrating the heme protein. The crude extract retains its cherry red CO color for as long as five months in the freezer at -20C, like the lupine. The extract, however, is contaminated with polysaccharide-containing material which responds positively to qualitative carbohydrate tests.

c. Spectrophotometric studies:

Pea and bur clover, like soy and lespedeza and the vertebrate hemoglobins, give spectra characteristic of protoporphyrin IX conjugates, including:

i. pyridine hemochromogen
ii. reduced alkaline hemochromogen
iii. cyanide hemi chromogen
iv. CO derivative

However, the crude heme proteins do not give the spectrophotometric response to pH change that soybean gives and that would permit a preview of the ionization which occurs on the water molecule attached to the iron atom (FeIII). The titrations between pH 6 and 10 are almost completely blocked. Our first guess is that the effect is physical, owing to contamination with the aforementioned gel. Another possibility, however, is that the hindrance is inherent in the protein structure itself. Final analysis must await purification of the heme proteins or chromatographic columns, etc.

Lupine crude extracts present the typical CO derivative spectrum. They await further study.

d. Acrylamide gel electrophoresis studies:

The Canalco technique: Studies have been made of soybean A and B, pea, bur clover, and lupine with this disk electrophoresis apparatus. The electrophoretic cell in this technique is a very small diameter glass tube. Reversals of migration are known to occur within the apparatus, and appear to be occurring with our material, which renders analysis difficult at this stage. Lacking photographic apparatus to record in color the pattern of the heme protein separations, our permanent records are the blue-stained disks which include many contaminant proteins in addition to the desired heme proteins. This renders a complex picture and the technique is being put aside for the time-being in favor of a macro technique.

The Canalco company has cooperated in our study, and has run samples for us. It has stated that our materials are very difficult to run, perhaps the most difficult proteins their staff has worked on.

The E-C Apparatus technique: The heme proteins have been tested repeatedly in the vertical cell technique, one and two dimensions, both discontinuous (disc) and continuous runs with satisfying results. The loan of a camera has permitted us to make permanent records of unstained (natural color) and stained gel slabs.

In general, the acrylamide gel separations mimic our paper electrophoretic separations of the heme proteins in question. This is important because many laboratories express little faith in paper separations, on which we have based our purification procedures in the past. The gel apparatus does indicate lowgrade contamination more readily than does the paper strip, and for this reason, checks between the two processes are valuable and we consider the gel an indispensable adjunct.

Soybean: There still appears to be no significant distinction between the Soy A and B heme proteins, nor between early and late nodules of Soys A and B. In other words, mere maturation of nodules has not contributed additional fragments of protein that can be discerned.

However, Soy A 1968 has a new component never seen before in our 18 years of experience with soybean grown with mixed commercial inoculum. This is a green component which migrates as a protein of the same molecular weight as the red fragments. It is not the usual green break-down product. The green component shows up on paper as well as in the gel. Soy B and the 1969 crop remain to be examined for this component.

Two-dimensional gel electrophoresis indicates that the heme proteins of soybean are of about the same molecular weight; it is not the case that certain of them are dimers, etc., nor fragments of low molecular weight. This statement applies to the 5-component extract, equally.

On the other hand, soybean-1960 heme protein preserved as the salt paste in the freezer over the years and rerun in 1969 shows a breakdown from an initial clear-cut three proteins to a well defined 5-component material. Both paper and gel bear this out. This is evidence that some of the components then are derived from others - not by cleavage into large fragments, but by loss of one or two amino acids, enough to permit a separation but not enough to decrease the molecular weight significantly. Evidence which supports this interpretation is found in the amino acid composition studies performed in 1968 which suggest that the minor soy components lack certain basic amino acids present in the major soy components.

The fact that Soy A and B heme proteins appear identical suggests that the bacteria responsible for nodule formation etcetera do not synthesize the protein moiety of the hemoproteins, since different strains of bacteria could be presumed to have different protein characteristics. This conclusion cannot yet be drawn. We require evidence that rhizobia A and B differ genetically in their protein constitutions.

Studies of rhizobia A and B: Dr. Joseph McGee of the University of Cincinnati Medical School and Veterans Administration Hospital has agreed to analyze strains A and B cultures as well as nodule-recovered strains A and B in the gas chromatograph, so that we may examine whether strain differences appear. (Ref: McGee, J. Characterization of mammalian tissues and microorganisms by gas-liquid chromatography. J. Gas Chrom. 6: 48-52, 1968.) The pure cultures are provided by Mr. Taylor of the Urbana Laboratories; the nodule-recovered bacteria come from our laboratory. It is essential to distinguish A from B, and to observe uncontaminated A and B recoveries from nodules before generalizing about sources of heme protein within the nodules.

Bur clover: The electrophoretic pattern of crude heme protein extracts is distinctive. There are four major heme protein components. One is more acidic than any of the soybean components, and migrates far ahead of the remaining three. The three migrate in a cluster at approximately the rates of the Fast and Slow main soy components. The four components, on the basis of 2-D gel finger prints, appear to be approximately of the same molelcular weight. The crude extract is heavily contaminated with foreign protein; 21 spots were picked up on the finger print as due to such contaminants. Bur clover extract is better handled on paper electrophoresis as a lower pH than soybean, and between 7 and 8 has given fairly clear separation. A combination of techniques, however, is going to be required to effect as clean a separation as is necessary. The soybean extract is incomparably cleaner, with regard to contaminants, than the bur clover.

Lupine: Lupine extract contains two major heme proteins and a minor fraction running slightly in advance. The pattern would not be confused with that of soybean because the two major components run close together and at a rate in between that of the soy Fast and Slow main. The extract is heavily contaminated with other proteins.

Pea: There appear to be three heme protein components in pea extracts. Satisfactory electrophoretic fractionation has not been worked out. There is suggestive evidence of a less acidic structure than characterizes the soy heme proteins.

e. Notes on non-heme components of the various extracts.

Ferredoxin: The brown color of several paper electrophoretic components in lupine, etc. has caused us to check on the possibility that ferredoxin is present in nodules. To date tests are negative. However, column separations will give cleaner fractions and testing will be repeated.

Yellow fluorescent components: All nodules examined have shown extraordinary yellow-green fluorescence under UV. The component, or one such component, involved is a slow migrating molecule preserved on paper electrophoresis strips. A few fractions have been preserved from column chromatography and one has been preserved from the free-flow electrophoresis obtained at Abbott Laboratories.

f. Amino acid compositions of heme proteins:

The Abbott Laboratories has pinpointed one difficulty encountered with their Technicon apparatus, on which they planned to run our amino acid hydrolysates. The apparatus does not give quantitatively proportionate results with different quantities of material applied to the column. They are working to correct the problem and promise to evaluate our heme proteins when satisfied their equipment is in working order. The correction is essential for our heme proteins, since they are so low in basic amino acids that larger amounts of hydrolysate must be applied than is required for the acid-neutral amino acids. It's impossible to correlate the results on the entire protein until the apparatus is corrected.

Meanwhile, the AAA Laboratory under the guidance of Hans Neurath is prepared to run samples for us, and checks should be made by this independent laboratory on our samples, since the liklihood of any other laboratory preparing our materials is small. In other words, the studies made on our materials should be double checked wherever possible to eliminate as much error in reporting as is humanly feasible.

g. Collaborative Study at Uttar Pradesh Agricultural University (India):

UPAU has undertaken to run field tests on nodulation of soybean planted 10 or 12 to the foot as opposed to 2 to 5 to the foot. It is a common observation in the United States that the thicker plantings, dear to our farmers, result in spindly plants, blown over in rain and wind, and in many plants lacking in nodulation. The question is raised whether the soybean seed yield will be the same with thinner planting, heavier stalks, and more abundant root and nodule development.

h. Training program:

We were asked to secure training for a professor from UPAU in the area of protein chemistry, amino acid analysis, and advanced laboratory technique. We arranged for an unusual and excellent training program involving: the AAA Laboratory in Seattle, Dr. Neurath's laboratory at the University of Washington, Dr. Margoliash's laboratory at the Abbott Laboratories, Dr. Nagy's laboratory at Loyola University in Chicago, and the Spinco Laboratory in Palo Alto. Funds have not been discovered to permit the individual to take advantage of the opportunity here. Rockefeller Foundation felt that India was adequately supplied with an amino acid analytical laboratory in New Delhi that the Foundation had provided. Information from UPAU, however, contends that the University is under pressure from plant growers to set up its own laboratory with its own personnel to handle studies on the quality of Indian grains. UPAU has on its own secured funds to purchase such equipment as the Spinco apparatus, an electron microscope, etc., out of its own agricultural program, and independent of the State of India. It is hoped resources will be discovered to permit the individual concerned to come and undertake the training arranged, since this training is unique and would not be available to him in India, nor can it be expected to be available indefinitely.

i. Anticipated Scientific Activity:

Sequential Analysis: The analysis will be conducted only on an incontestably homogeneous heme protein which must be available in about one gram quantity at the outset. This is expected to be a soybean component. The lespedeza still is not available in sufficient quantity. Pea, lupine, and bur clover are too difficult to purify in large quantity even if they were collected in large quantity.

The analysis can be conducted stepwise, but no step should be taken without assurance that the step can be completed within the funding-life of the program. The following introductory steps are possible in initiating the sequential analysis:

i. Obtaining and purifying one gram of heme protein and immediately hydrolysing it into peptides with trypsin. Freeze and store.

ii. Pass the entire hydrolysate through column chromatography, collect the (test tube) effluent, ascertain the ninhydrin peaks, and combine the test tube fractions accordingly. Store and freeze.

iii. Concentrate (i.e. lyophilize or flash evaporate) the foregoing fractions.

iv. Singly, exame the finger prints of the fractions and determine the number of peptide or amino acid spots, and how to best separate them individually.

v. Separate the above and store, frozen.

vi. Run amino acid hydrolysis and analysis on each peptide previously isolated.

With a methodical numbering system (essential to the process) the protein can thus be digested and stored at various stages of study.

Hydrogenase activity: Nodule material is available for biological assay of hydrogen fixation: bur clover, lupine, pea, vetch, sweet pea, and lespedeza, all 1969 samplings.

Spectrophotometric analyses: Spectrophotometric titration of the water molecule attached to the FeIII atom should be determined on fractions from bur clover, lupine, and pea if the latter is available in sufficient amounts.

j. List of contributors to our program the past year:

Universities:

University of California
Botanical Garden
Mr. W. Roderick
Mr. A. Christ, Manager

U. of Cincinnati Medical School
Dr. Joseph MacGee

Uttar Pradesh (India) Agricultural University
School of Basic Science and Humanities
Dr. K.G. Gollakota, Director

Companies:

Abbott Laboratories, Protein Section
Dr. O. Walasek
Dr. E. Margoliash, Section Head

The Urbana Laboratories
Mr. Wm. M. Taylor, President

Souverain Cellars
St. Helena, California
Mr. L. Stewart, Owner

Farmers:

Mr. Don Myrick
Rantoul, Illinois (RR1, zip 61866)

Mr. John Doyle,
Caribou, Maine