* The author's work was done under two basic research grants awarded to him by the National Science Foundation.

[1] Pearson, E. H., and H. S. Vandiver, "On a New Problem Concerning Trinomial Congruences Involving Rational Integers," these PROCEEDINGS, **39**, 1278–1285 (1953); Lehmer, Emma, and H. S. Vandiver, "On the Computation of the Number of Solutions of Certain Trinomial Congruences," *Jour. of the Assoc. for Computing Machinery*, **4**, 505–510 (1957); Vandiver, H. S., "The Rapid Computing Machine as an Instrument in the Discovery of New Relations in the Theory of Numbers," these PROCEEDINGS, **44**, 459–464 (1958). We shall refer in the present paper to the above as papers I, II, and III, respectively.

[2] Nicol, C. A., J. L. Selfridge, and H. S. Vandiver, "On Diophantine Equations Which Have No Solutions," these PROCEEDINGS, **42**, 264–266 (1956).

[3] Takagi, T., "Ueber eine Theorie des relativ-Abelschen Zahlkörpers," *J. Coll. Sci., Imp. Univ. Tokyo*, **61**, 16 (1920).

[4] If the left-hand member of (7) is a unit, then (7) still holds, since then α is the unity ideal.

[5] Due to Gauss for $l = 3$. It is included as a special case of a relation given by Mitchell, H. H., *Proc. Amer. Math. Soc.*, **17**, 167 (1916), involving the number of solutions of trinomial equations in finite fields.

[6] Vandiver, H. S., "New Types of Trinomial Congruence Criteria Applying to Fermat's Last Theorem," these PROCEEDINGS, **40**, 250–251 (1954).

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*SPECTROPHOTOMETRIC STUDIES OF THE EFFECTS OF NITROGEN ON SOYBEAN NODULE EXTRACTS**

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Shug, Hamilton, and Wilson[1] and Hamilton, Shug, and Wilson,[2] demonstrated changes in the absorption spectra of sonic extracts of *Clostridium pasteurianum*, *Azotobacter vinelandii*, and soybean root nodules, when the extracts were placed under atmospheres of He, H_2, and N_2. With the nodule extracts these changes corresponded to the reduction and oxidation of the hemoglobin component. This paper presents data which support the specificity of the hemoglobin oxidation in the presence of N_2 and demonstrates the ability of the bacterial component of the nodules to reverse this effect.

Materials and Methods.—The methods used were based on those of Hamilton, Shug, and Wilson[2] and all precautions detailed by these authors were observed. The following details differ from those previously described. Nodules aged 4–5 weeks from glasshouse grown Lincoln variety soybeans inoculated with Strain CC711 of *Rhizobium japonicum* were used. Attempts to use nodules frozen and stored under H_2 were not successful, and extracts were prepared within one hour of nodule harvest, by crushing 2–3 gm. under a stream of H_2 in $M/15$ phosphate buffer pH 7.0 saturated with H_2. All manipulations were done at temperatures between 0° and 4°C. The crushed material was placed in the sonic field of a Raytheon 10-kc magnetostrictive oscillator for 3–5 minutes in a H_2 atmosphere. Longer sonic treatment reduced the activity of the extracts. After centrifugation at 7,000 × *g* for 15 minutes in tubes closed under a stream of H_2, the extracts were immediately transferred to 1 ml or 3 ml Pyrex cuvettes of 1 cm light path, fitted with Thunberg caps, and quickly evacuated. The gas space was then flushed ten times with

scrubbed He to remove the last traces of air. Storage of these preparations at $-20°$ under H_2 or He resulted in rapid loss of activity with respect to N_2, although the H_2 effect remained after 24 hours storage. Consequently all the experiments reported here were completed within 2 hours of the removal of nodules from the plants.

Commercial high purity helium, hydrogen, and nitrogen were used after being freed from contaminating O_2 by scrubbing with a solution of chromous chloride: (25% w/v $CrCl_3$ in 2*N* HCl reduced by adding Zn dust in an atmosphere of N_2 in a flask; when fully reduced the chromous chloride solution was transferred under vacuum to the scrubbing towers). In addition to scrubbing, N_2 was stored over chromous chloride solution in an aspirator bottle for at least 24 hours before use. Vessels were evacuated and filled from a vacuum line equipped with a mercury manometer and permanently connected with the gas supplies through scrubbing towers. Between manipulations all gas lines were flushed ten times with scrubbed He.

Gas mixtures containing small amounts of O_2 were obtained with a capillary gas buret calibrated in hundredths of a milliliter attached to a 2 liter bulb and connected to the vacuum line. Air in the buret was added to the scrubbed gas in the bulb by raising mercury in the capillary.

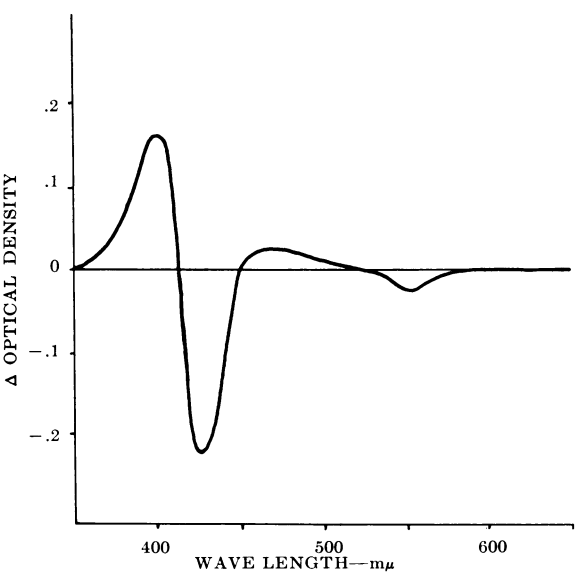

Fig. 1.—Difference spectrum of soybean nodule sonic extract. Reference cuvette He, sample-scrubbed N_2. The baseline was obtained with both cuvettes containing scrubbed He.

Spectra of clear extracts were obtained with a Beckman model DK2 ratio recording spectrophotometer and with a Beckman Model DU for extracts to which suspensions of bacteroids were added. In all cases the absolute and difference spectra between cuvettes were obtained for extracts under He. Preparations which contained more than a trace of hemiglobin were discarded, as also were preparations whose He—He difference spectra departed markedly from a straight

line. Slight deviations were compensated by replotting the curves obtained after changing the gas atmosphere.

Here, as elsewhere (Bergersen[3]), the term bacteroid is used to denote the intracellular forms of nodule bacteria which fill the central tissue of nitrogen-fixing nodules. Suspensions of bacteroids were obtained as previously described[3] from crushed nodules and washed in five changes of $M/15$ phosphate buffer pH 7.0.

Results.—Active nitrogen-fixing nodules yielded sonic extracts in which the hemoglobin was oxidized in the presence of scrubbed N_2 (Fig. 1). Occasional inactive preparations were attributed to the use of nodules older than 6 weeks, failure to maintain adequate exclusion of air during preparation and undue delays between steps. The difference spectra obtained between N_2 and He reached maximum values within 5 minutes from the final N_2 flushing and agitation(Table 1). When preparations oxidized in this way were flushed with H_2, the difference spectra indicated reduction of the pigment to give a more reduced form than in the preparation under He. Extracts allowed to stand for 4 hours under gas atmosphere containing 25 p.p.m. O_2 showed considerable oxidation of the pigment to hemiglobin due to the action of polyphenol oxidase in the preparation. Flushing these oxidized extracts with hydrogen had no effect.

TABLE 1

THE DEVELOPMENT OF THE N_2—He DIFFERENCE SPECTRUM OF SOYBEAN NODULE EXTRACTS

Wavelength, mμ	Δ OD at 1 min.	3 min.	5 min.	10 min.
400	+0.042	+0.110	+0.112	+0.113
425	−0.050	−0.110	−0.113	−0.113
555	0.000	−0.010	−0.018	−0.018

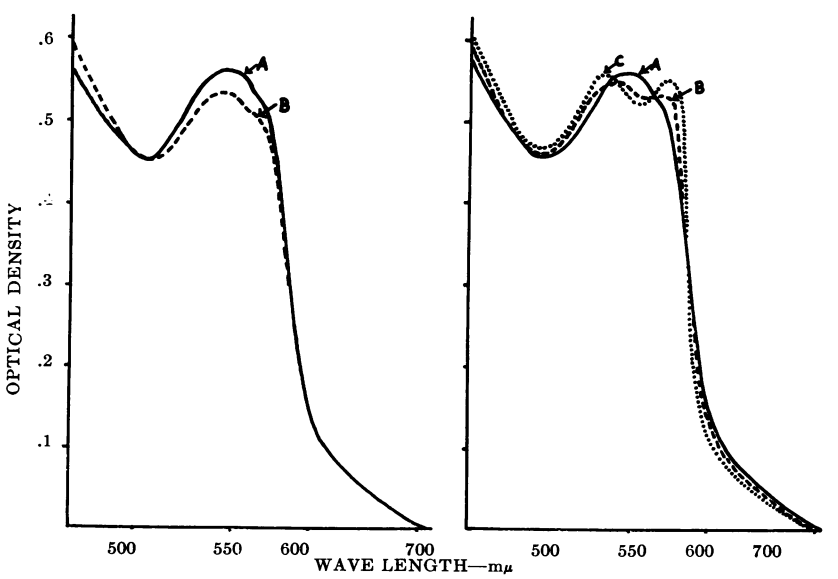

Fig. 2.—(*a*) Curve *A*, spectrum of nodule extract under He; Curve *B*, spectrum of the same extract under scrubbed N_2 5 minutes after the gas change.
(*b*) Curve *A*, spectrum of extract under He; Curves *B* and *C*, extract under He containing 8 ppm and 20 ppm O_2, respectively, 5 minutes after the gas change.

As is illustrated in Figure 2, the use of scrubbed He, to which traces of air had been added, to replace the scrubbed He over the extracts in the cuvettes, showed, that in the first 10 minutes after changing the gas, the hemoglobin reacted with the oxygen to form oxyhemoglobin and not hemiglobin, as shown by the development of peaks at 560–565 mμ and 540 mμ, (Sternberg and Virtanen[4]).

No activity with respect to N_2 could be detected in extracts from which the sonic step had been omitted, and sonic treatment for 20 min. also produced inactive preparations. All extracts, however, contained hemiglobin after standing for 4 hours under atmospheres containing traces of added air, irrespective of presence or absence of activity with N_2.

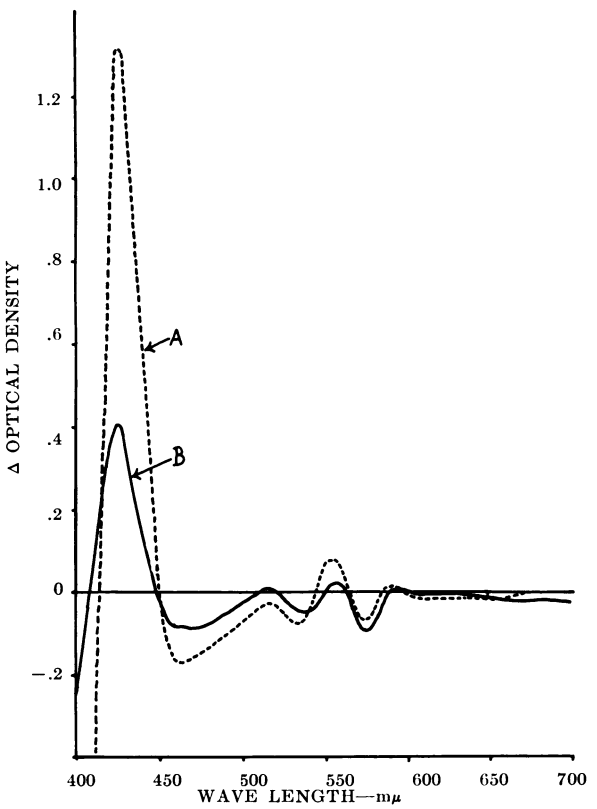

FIG. 3.—Difference spectra (reduced-oxidized) of soybean nodule extracts. *A*, oxidized with air and reduced with dithionite. *B*, oxidized with air reduced with bacteroids under an atmosphere of He. Both cuvettes contained 2.8 ml nodule extract and 0.2 ml of bacteroid suspension; the reference cuvette was bubbled with air between readings and the sample cuvette was filled with He. The baseline is the difference spectrum before addition of dithionite for *A*, and change of air to He in *B*.

When washed bacteroids were added to an air oxidized preparation from which air had been replaced by He the hemiglobin was rapidly reduced as shown in Figure 3. Preparations capable of showing oxidation of hemoglobin with N_2 failed to do so when washed bacteroids were added from the Thunberg cap prior to the change in the gas atmosphere. Thus, bacteroids are capable of reducing hemiglobin to hemoglobin and of reversing the oxidation produced by N_2 in active preparations.

Discussion.—The observations herein reported add to the arguments of Hamilton, Shug, and Wilson[2] for the validity of the N_2 effect on sonic extracts of soybean nodules. These workers could detect no O_2 in their N_2 samples in the mass spectrometer and concluded from the specificity of the reactions with H_2, He and N_2, that O_2 was not involved in the oxidation observed with N_2. The observation that traces of O_2 as low as 8 p.p.m. in He, produced oxygenation of the hemoglobin in the preparations within the same limits of time in which N_2 produced oxidation of the pigment, support these conclusions. The absence of activity from extracts not exposed to the sonic treatment and from extracts subjected to 20 minutes of sonic treatment, lends support to the conclusion that active preparations contain fragments of the nitrogen fixing complex. These fragments are capable of binding nitrogen and accepting hydrogen from hemoglobin. The observations of Hamilton, Shug, and Wilson[2] that H_2 produces the reverse effect, reducing hemiglobin to hemoglobin may mean, as suggested by Winfield,[5] that hydrogenase in nodules acts as a hydrogen transferase between a hydrogen carrier and the activated bound N_2, and may in fact be part of the nitrogen fixing complex.

The observation reported by Appleby and Bergersen[6] that bacteroids could reduce hemiglobin under anaerobic conditions have been extended here to show a possible role of hemoglobin as a coupling agent between the terminal respiratory pathways of the bacteroids and the nitrogen-fixing site.

Bergersen and Briggs[7] demonstrated a system of membrane envelopes, within the host cytoplasm, enclosing groups of bacteroids. From cytochemical tests for heme iron, it appears that hemoglobin in soybean nodules is located within these membrane envelopes, (Bergersen, unpublished). Bacteroids are capable of high rates of O_2 uptake (Bergersen[3]) and since the dimensions of the envelope-system indicates about 10^4 envelopes per host cell, each containing at least four bacteroids, O_2 tension would appear to be very low indeed. Under these conditions it is possible that the hemoglobin becomes an extracellular part of the terminal respiratory pathway of the bacteroids, which in nitrogen fixing nodules, may well terminate with the reduction of atmospheric nitrogen. This type of pathway has already been suggested by Parker,[8] and may be summarized as follows: bacteroid respiration→hemoglobin→nitrogen-fixing complex.

The use by Hamilton, Shug, and Wilson[2] of nodules frozen under H_2 for the preparation of active extracts could not be repeated. Only extracts prepared from freshly harvested, actively N_2-fixing nodules were capable of producing hemoglobin oxidation in the presence of N_2, although some frozen nodule preparations showed shifts of absorption spectra in the presence of H_2. It is well known that nodules cease to fix nitrogen after such treatments as freezing and this again tends to support the specificity of the reactions under discussion.

Summary.—The ability of sonic extracts of fresh, active soybean nodules to react with gaseous N_2 to produce oxidation of hemoglobin in the preparation, has been confirmed, and the reaction has been shown to be complete within 5 minutes. The possible role of traces of contaminating O_2 in the N_2 in producing the effect has been eliminated by the demonstration that traces of O_2 first cause measurable oxygenation, and not oxidation of the hemoglobin. Oxidation of the pigment in the preparation follows after standing for several hours.

The ability of bacteroids to reduce oxidized hemoglobin has been demonstrated

and the possible role of the pigment as a hydrogen carrier between the terminal respiratory pathway of the bacteroids and the nitrogen-fixing complex is discussed.

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[4] Sternberg, H., and A. I. Virtanen, *Acta Chem. Scand.*, **6**, 1342–1352 (1952).

[5] Winfield, M. E., *Revs. Pure Appl. Chem.* (Australia), **5**, 217–246 (1955).

[6] Appleby, C. A., and F. J. Bergersen, *Nature*, **182**, 1174 (1958).

[7] Bergersen, F. J., and M. J. Briggs, *J. Gen. Microbiol.*, **19**, 482–490 (1958).

[8] Parker, C. A., *Nature*, **173**, 780 (1956).

ERRATA

In the paper "Optical, Electromagnetic, and Satellite Observations of High-altitude Nuclear Detonations—Part I," appearing in these PROCEEDINGS, **45**, 1208–1221 (1959), the two sentences beginning on line 22 of page 1215 should read as follows:

"A height of 100 km for the aurora at a range of 800 km requires an elevation angle of 3° 20′, apparently below the visual aurora. In this case, the ground conjugate point is deviated by about 200 km to the northwest of the conjugate point for event III computed at the RAND Corporation by Vestine and Karzas."

The reference number on line 9 of page 1215 should be 2, not 13.

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