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Hemoprotein from Root Nodules and Nitrogen Fixation by *Rhizobium*.*

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Although the necessity of root nodule bacteria for fixation of molecular nitrogen by leguminous plants has been established since 1886, experimental proof that these organisms are the responsible agent still is lacking. In spite of numerous claims of fixation by pure cultures of the bacteria, few such claims withstand critical examination.[1] The identification of the red pigment in root nodules as a hemoprotein[2-5] led inevitably to the suggestion it was directly concerned with the fixation reaction. Among the support for this proposal was an experiment by Virtanen and Laine[6] in which addition of nodular extracts to pure cultures of *Rhizobium* apparently enabled them to fix appreciable quantities of nitrogen. The fixation was particularly striking if oxalacetic acid was added with the extract. This claim appeared surprising in view of the difficulty in inducing excised nodules to assimilate molecular nitrogen although they still contain both bacteria and pigment. Nevertheless, because of its obvious importance, extensive tests of the possibility were undertaken. Such tests are limited to the summer months when sufficient nodules can be grown to provide pigment for extensive replication. During the past 2 summers we have made these tests but have obtained no evidence that the presence of the pigment or any other constituent of the extract of nodules from soybean or pea stimulate the free living *Rhizobium* to fix nitrogen. Since these experiments were completed, a new report from Virtanen[7] states that more extensive trials fail to confirm their first experiment; we shall summarize here only the experimental variations tested and the results.

Materials and Methods. Nodules were taken from leguminous plants grown on a nitrogen-poor sand in a cold-frame. Twice during the growing season the plants were watered with Hoagland's N-free nutrient solution. The nodules were picked into cold water, drained, and macerated with an equal quantity of water. The extract was pressed through cheesecloth, centrifuged for 20 minutes, then passed through a Berkefeld *N* filter and suitable aliquots added to 6 oz prescription bottles containing 25 ml N-free medium. This medium was: Allison's salt mixture, 1.5 g; sucrose, 10 g; water, 1000 ml. Traces of Fe and Mo were supplied together with 1 ml/liter yeast water (2 mg N) for growth factors. When the soybean organism was used, 1 ml of a mesquite gum hydrolysate was also added. Various species of *Rhizobium* were grown on agar slants (Medium 79 of Fred and Waksman), and aliquots of their suspensions added to the N-free medium. The incubation was at 30°C for 7 days; total nitrogen was determined by a semimicro Kjeldahl procedure sensitive to 0.02 mg.

Experimental variations tested in attempts to secure fixation included:

1. *Source of nodules.* Soybeans inoculated with *Rhizobium japonicum* 534 and Canada field peas with *R. leguminosarum* 317 were used. Nodules from plants 6 to 9 weeks old

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TABLE I.
Summary of Nitrogen Fixation Experiments.

Treatment	Exp. I		Exp. II		Exp. III	
	No. of samples	Mean N, mg/25 ml	No. of samples	Mean N, mg/25 ml	No. of samples	Mean N, mg/25 ml
Control	2	2.95	2	2.57	2	2.65
None	2	2.96	3	2.62	3	2.63
α-Ketoglutarate	2	2.96	4	2.51	4	2.72
Oxalacetate	2	2.99	3	2.60	4	2.64
Citrate	2	3.03	4	2.66	3	2.73
	Exp. IV		Exp. V		Exp. VI	
Control	3	3.25	2	1.63	2	3.38
None	4	3.24	—	—	—	—
α-Ketoglutarate	3	3.25	2	1.66	2	3.27
Oxalacetate	2	6.37*	2	1.38	3	3.73
Citrate	3	3.19	2	1.69	2	3.25
	4	6.38*				
	Exp. VII		Exp. VIII		Exp. IX	
Control	2	2.41	2	3.20	2	2.64
None	2	2.49	2	3.19	3	2.91
α-Ketoglutarate	4	2.43	3	3.36	2	2.99
Oxalacetate	3	2.38	3	3.22	—	—
Citrate	2	2.45	3	6.14*	2	2.94

Extract of nodules containing pigment added to all samples; those marked with asterisk given twice the level of others. Controls kept at 3°C, others at 30°C.

gave the most satisfactory extracts.

2. *Method of Extraction.* In about one-half the experiments, the nodules were extracted in the cold under carbon monoxide. Before its addition to the medium the pigment was reoxygenated for 20 minutes.

3. *Filtration.* The finest filter tried was a Seitz, but a Berkefeld *N* was usually used as it did not clog. Extracts of nodules from plants in the flowering stage were so viscous that they passed only through the cheesecloth.

4. *Organism.* Species of *Rhizobium* tested, singly or in mixtures were *R. japonicum, R. leguminosarum, R. melilotii,* and *R. trifolii.*

5. *Additions.* Various organic acids, oxalacetic, α-ketoglutaric and citric, often implicated in fixation schemes were added (15 mg/25 ml).

6. *Incubation.* In some experiments the bacterial cells were incubated for 3 days in the N-free medium before the addition of the extract as was suggested by Virtanen and Laine;[6] in others, the cells and extract were added at the same time.

7. *Miscellaneous.* Two to 4 replicates were made of each treatment. The controls were treated in the same way as the experimental flasks but were kept at 3°C. The quantity of pigment added varied with the preparation, but an attempt was made to standardize this so that the extract added to 25 ml contained 2 to 3 mg N. In 2 experiments twice the usual level of extract was supplied. About 0.1-0.2 mg N was contained in the bacteria used as inoculum.

Results. The agreement in nitrogen content among replicates is illustrated by the data from a typical experiment: Control, 2.60, 2.53; plus extract, 2.62, 2.61, 2.63; plus extract and oxalacetate, 2.58, 2.58, 2.62; plus extract and α-ketoglutarate, 2.66, 2.50, 2.63, 2.62; plus extract and citrate, 2.55, 2.72, 2.58, 2.69 mg per 25 ml. Statistical analysis of the data from 9 experiments (113 samples) indicated that the standard deviation of the means of duplicates was about 0.15 mg and of the means of quadruplicates about 0.10 mg. Therefore, the means of the replicates for each treatment would have to exceed that of the control by 0.2 to 0.3 mg even to approach statistical significance. Actually, of course, one would wish greater differences for so important a conclusion, *e.g.,* a gain of at least 0.5 to 1 mg of N per bottle. As can be seen

by the summary in Table I no such gains were obtained. It is concluded that if fixation occurs it is too weak to be detected by the Kjeldahl method. The possibility that the slight gains occasionally observed possess significance is now being tested using the much more sensitive isotopic method.

Summary. Fixation of molecular nitrogen could not be induced in free-living cultures of *Rhizobium* by supplying them with extracts of root nodules containing the hemoglobin-like pigment. Nine experiments were made during 2 growing seasons. Variations in technique included: species of bacteria; source of nodular extract; addition of oxalacetic, α-ketoglutaric and citric acids; and method of preparing the extract. Analyses of the results showed that gains of 0.2-0.3 mg N were required for statistical significance, but even these slight increases were not consistently obtained.

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Effect of Induced Liver Cirrhosis on the Reproductive System of the Male Rat.*

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While it has been shown repeatedly that the physiology of reproduction is dependent upon the maintenance of a normal pituitary-gonadal balance, it is also becoming increasingly evident that intact liver function is essential to the normal metabolism of the sex steroid hormones. The results of *in vitro* experiments performed by numerous investigators have provided ample evidence that the liver is the principal organ responsible for the inactivation and removal from the blood of the natural estrogen of the body (Silberstein *et al.*,[1] Zondek,[2] Engel and Navratel,[3] Heller *et al.*,[4] Heller,[5] and Engel,[6]). Inactivation of endogenous estrogens by the liver has been reported by numerous workers using varying techniques. Talbot[7] induced liver damage in female rats with carbon tetrachloride in alcohol and found an increase in uterine weight of 200% by the third day of the experiment. From this he concluded that the poisoned animals were exposed to an increased concentration of blood estrogen because of the impaired inactivating capacity of the liver.

Other experiments (Golden and Severinghaus,[8] Biskind and Mark,[9] Biskind,[10] Krichesky, Benjamin and Slater,[11]) were devised to divert the gonadal hormones directly into the portal or into the systemic circulation. When the portal route was used the hormones were promptly inactivated, as shown by failure to maintain the sex accessories of castrate animals, whereas the hormones escaped inactivation when they drained directly into

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