Technical Papers

Time Course of Fixation of N_2 by Excised Soybean Nodules[1]

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Experimental difficulties have been encountered by those seeking to demonstrate fixation of N_2 by excised nodules of leguminous plants. The earlier reports, reviewed by Wilson (*1*), and Allison, Hoover, and Minor (*2*), were based on gasometric analysis or analysis of nitrogen by the Kjeldahl method. As neither technique is particularly sensitive, the nodules were kept for relatively long periods before analysis; the possibility of fixation of N_2 by nonsymbiotic bacteria during such extended incubation could not be excluded. Much more sensitive and specific tests for fixation can be made with $N_2{}^{15}$ as a tracer, but even such tests have been erratic, and the suspicion of fixation by contaminating organisms has remained (*3*).

Lincoln soybeans inoculated with *Rhizobium japonicum* were planted in the field and grown until nodules 3–5 mm in diameter were formed. The plants fixed N_2 vigorously, and the interior of the nodules was red with hemoprotein. Intact plants with the soil undisturbed around their roots were brought to the laboratory. The soil was dislodged from a few plants at a time, and their nodules were quickly removed and placed in Warburg flasks containing 0.5 ml of water. The flasks were attached to Warburg manometers, and 2 manometers at a time were quickly evacuated (*4*), flushed twice with He, and then filled with a mixture of 65% He, 20% O_2, 5% CO_2, and 10% N_2; the N_2 had approximately 20 atom % N^{15} excess. Three to 5 min elapsed from the time the nodules were removed from the plant until they were gassed with $N_2{}^{15}$. The flasks were shaken in a bath at 20° C, and pairs of flasks were removed at intervals. The nodules were ground immediately in a glass mortar with a few ml of 3 *N* HCl, centrifuged, and then washed with water by centrifugation. The combined extract and washings were subjected to Kjeldahl digestion, and the insoluble residue of the nodules was discarded. The ammonia in the digest was converted to N_2 and was analyzed for N^{15} with a mass spectrometer.

The data from 4 experiments, including 34 individual determinations, are summarized in Fig. 1; the values plotted are the averages of all the determinations at each time interval. Fixation of N_2 was obtained consistently, for only 1 sample (20-min harvest) had less than 0.05 atom % N^{15} excess. There was

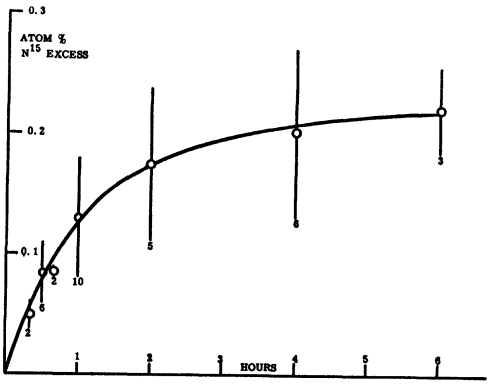

FIG. 1. Fixation of $N_2{}^{15}$ into the soluble portion of soybean root nodules. Averages from all determinations are plotted. The vertical lines represent the standard deviation in the analytical results; the number of samples at each time interval is given at the base of these lines.

considerable variation among samples, as emphasis was placed on speed of treatment rather than on selection of nodules for uniformity; however, the averages define a smooth curve when fixation is plotted against time (Fig. 1). After 6 hr the fixation was less than twice that at 1 hr. The decrease in rate of fixation of N_2 with time suggests that an intermediate necessary in the fixation process was being depleted or that a necessary enzyme was being inactivated. In limited trials, the addition of 0.01 *M* solutions of neutralized (pH 6.2) α-ketoglutaric, oxalacetic, citric, acetic, succinic, or malic acids did not support fixation appreciably different from that observed with water only.

That the observed uptake of N^{15} was not the result of fixation by contamination organisms is attested by the speed of the fixation. More important, the rate of fixation decreased with time, whereas one would anticipate that fixation by contaminating agents should remain linear or increase with time.

The consistent fixation of $N_2{}^{15}$ observed in these trials is attributed to: (*a*) the use of field-grown plants which usually fix N_2 more vigorously than those grown in the greenhouse; (*b*) the rapid exposure of the nodules to $N_2{}^{15}$ after excision; (*c*) the analysis of only the soluble portion of the nodules. With conditions defined for obtaining consistent fixation, tests can be performed on the symbiotic N_2-fixing system without the difficulties attending the use of intact plants. Problems concerned with the nature of the materials supplied the nodule by the plant, and the influence of temperature, pH, pO_2, and pN_2 on fixation are all open to a simplified experimental approach.

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A Saliva Test for Prenatal Sex Determination

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During the course of investigating some of the many ramifications of the Richardson Pregnancy Test (*1*) two rather interesting observations were made. The Richardson test depends upon the presence of free estrone, in contrast to bound or modified estrone or similar 17-ketosteroids in the female urine. This level of free estrone substances rises sharply soon after conception, the test being positive in as little as 2 weeks after conception, sometimes even before the first missed menstrual period. We were naturally interested in determining whether the free estrone level rose in other body fluids. Blood and urine have already been mentioned in Richardson's original article. We investigated such fluids as saliva, tears, and perspiration. The studies upon saliva yielded the results that are the subject of this report.

It was noted early in the study that in only some of the women who were in their sixth or seventh month of pregnancy did the Richardson test prove positive when the saliva was tested. In each of these cases, however, the test was positive on the urine. The apparent answer to the problem was forthcoming only after the delivery of the child. In nearly every case, the positive tests were associated with a male child, and most of the negative tests were associated with a female child. A detailed study followed. The results are presented in Table 1.

TABLE 1

RELATION BETWEEN SEX OF CHILD AND REACTION OF THE MOTHER'S SALIVA TO THE RICHARDSON TEST

	Positive	Negative
Males	218	3
Females	7	148

The precise nature of the substance responsible for the positive test is not known. It is believed that some androgenic substance is being identified, since, whereas a nongravid female normally yields a negative test, after the injection of testosterone or androsterone a strongly positive reaction results. The male saliva, spermatic fluid, and blood serum are all strong positive reactors.

The selective excretion of certain blood constituents through the salivary gland is well known. These findings illustrate a rather delicate selectivity of female salivary glands in their capacity to screen out certain female-associated hormones, but to allow certain male-associated ones to pass into the salivary fluid.

A detailed report of this project will be published in suitable medical journals. Because of their obvious practical nature, these findings are preliminarily reported here to allow early verification by others.

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Influence of Cobalt on Reproduction of Mice and Rats

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Reproduction studies with mice and rats kept on whole plant rations have been in progress in this laboratory for several years. In the first experiments, poor reproduction performance was observed (*1*), but later much more satisfactory results were obtained with a slightly modified diet (*2*). The only difference in the composition of the diets was that the salt mixture supplement used originally did not contain cobalt, whereas in the later experiments 0.2% $CoCl_2$ was added to the salt complement. Because of the apparent influence of this small amount of dietary Co on reproduction, the present study was undertaken.

The rats used were of the Sprague-Dawley strain, raised in our laboratory; the mice were of the same albino strain used in the previous experiments. The rats had been kept on a whole plant ration for at least 3 generations prior to the start of the present experiments, and the mice for at least 5 generations. The animals were kept on the basal ration in common cages until females became pregnant. These were put in single screen-bottomed cages and given the respective experimental diets and water *ad lib.* Litters were reduced by random selection to 6 and weaned at the age of 28 days.

The composition of the basal diet was: solvent-extracted soybean meal, 46; cornmeal, 46; sesame oil containing 0.2% percomorphum oil and 0.2% wheat germ oil, 5; salt mixture II USP, 2; thiamine, 0.3 mg; riboflavin, 0.3 mg; calcium pantothenate, 2 mg; pyridoxin, 0.2 mg; choline hydrocloride, 100 mg; nicotinic acid, 2 mg; folic acid, 0.025 mg; biotin, 0.01 mg; inositol, 10 mg; PABA, 25 mg. The diet has been analyzed by microbiological methods and found to contain about 0.5 μg/100 g of vitamin B_{12} activity (*2*). Cobalt was supplied in the diet by adding 0.2% $CoCl_2$ to the salt mixture, or in the drinking water in a concentration of 0.2 mg% of $CoCl_2$; when cobalt was injected, a 0.1% solution of $CoCl_2$ in physiological saline solution was used, of which 0.2 ml was injected in each female 2–6 days prior to giving birth. A total of 130 litters of mice and 64 litters of rats was used in the present study.