phoric acid was used yielded fluorescent products with sulfuric acid.

A detailed study of metabolism of estrogens in normal, pregnant and pathological conditions will be published elsewhere.

Summary. 1. A quantitative method for the determination of estradiol, estrone and estriol in urine, based on the fluorescence of these substances when treated with phosphoric acid, is described. 2. The method is more accurate, sensitive and specific than other known biological or colorimetric tests. 3. The recoveries of estradiol were above 80%; of estrone between 70 and 80%; and of estriol between 50 and 60%. The mean experimental error was ± 10%. 4. The described method permits the determination of estriol in urine in quantities not hitherto detectable.

The author wishes to express his indebtedness to Prof. Bernhard Zondek for his kind interest and support of this investigation.

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Isotopic Studies of Fixation by Rhizobia in Presence of Hemoprotein.*

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Claims of fixation by free-living species of *Rhizobium* in the presence of plant extracts, growth factors and other organic supplements frequently have been made but not verified.[1] The most recent, that hemoprotein from the root nodule can induce fixation *in vitro,* was suggested by the observation that the pigment occurs in the red state during active fixation but disappears from ineffective or old nodules. Although attempts to substantiate the positive experiments failed,[2,3] such negative findings are not necessarily critical. Fixation by pure cultures might be stopped by the accumulation of an intermediate, and pure cultures, long cultivated on combined nitrogen in the laboratory, might lose their ability to assimilate molecular nitrogen. These objections can be overcome by testing for fixation with the stable isotope, N^{15}, and by using species of *Rhizobium* taken directly from the nodules. Whereas the conventional Kjeldahl method requires fixation of 1.0 mg or more for positive claims, the isotopic method readily detects fixation of as little as 0.01 mg and practically eliminates sampling errors.

Materials and Methods. By "pigment" is meant an aqueous extract from nodules actively fixing N_2 and red with hemoprotein. This extract, after filtration through cheesecloth and centrifugation in a Beams centrifuge, is sterilized by passage under pressure through a Seitz filter. The preparations are usually gummy and difficult to filter. In initial experiments with such filtrates fixation was obtained. In agreement with our earlier results[2] the increases in nitrogen content were too small to have been detected by the Kjeldahl method, but were definite with the isotopic technic. Microscopic and cultural tests revealed a large number of organisms, primarily the root nodule bacteria. Inability to secure sterile filtrates did not arise from faulty technic or poor filters since no difficulty was experienced with laboratory cultures of the rhizobia. These observations suggest a sub-microscopic filterable form in the nodule (Almon and Baldwin).[4] We believe, however, it is more likely that the effect

* Supported in part by grants from the Rockefeller Foundation and from the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

1 Wilson, P. W., *The Biochemistry of Symbiotic Nitrogen Fixation,* University of Wisconsin Press, 1940.

2 Niss, H. F., and Wilson, P. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 233.

3 Virtanen, A. I., Jorma, J., Linkola, H., and Linnasalma, A., *Acta Chem. Scand.,* 1947, **1**, 90.

TABLE I.
Summary of Isotopic Nitrogen Fixation Experiments.

Exp.	Source of pigment	Cultures	Atom % excess N^{15}		
			Initial gas	Inoculated	Uninoculated
	Sterile				
1	Soybean, cowpea	534	8.85	.010	—.001
2	Cowpea	317, 618	10.20	.011	
		Azotobacter		3.5	
		Clostridium*		.36	
		Radiobacter		.009	
		Mixture†		.90	
3	Soybean	317 and			
		Radiobacter	10.80	.010	
4	”	317, 610, 534		—.016	
		and Radiobacter	10.60	—.016	
5	”	Clostridium*	8.73	.013	
				.011	
	Nonsterile				
6	Soybean	534	7.88	.045	.062
				.151	.053
7	”	317, 534	2.84	.040	.048
				—.009	
				—.001	
				—.020	
8	Cowpea	317	8.15	—.001	—.001
				—.002	—.004
9	”	610	9.40	.012	.014
10	Soybean		10.06		.076
					.076

Increase of 0.050 atom % excess necessary for significant gain.
* Isolated from a filtered but nonsterile nodular extract.
† Mixture of all cultures used in experiment.

of the gum on the physical properties of the filter determines whether a given organism is retained since suitable dilution of the gummy preparation resulted in a sterile filtrate.

This curious filtering property of the root nodule bacteria was unexpected but useful as it provided a convenient method for securing a culture of the organism directly from the nodules for test of its ability to fix nitrogen. The uncertainty of whether other contaminating forms likewise accompanied these "filterable" rhizobia necessitated suitable bacteriological observations for control of the population in such nonsterile cultures. Microscopic stains were made at every stage of the preparation of the pigment to note the decrease in numbers of organisms, and cultural tests were made on the contents of the isotopic flasks at the end of the experiment. *Azotobacter* was tested for by inoculation of Burk's nitrogen-free medium, *Clostridium,* by inoculation of Winogradsky's nitrogen-free medium and incubating in an atmosphere of molecular nitrogen; and *Rhizobium* by plating on yeast water-mineral salts—sucrose agar. All media were incubated for at least two weeks before discarding; if any growth occurred, a microscopic examination was made; if fixation, the culture was further identified.

The species of *Rhizobium* used were: pea culture 317, soybean culture 534, cowpea cultures 610 and 618. Both positive and negative controls were obtained by including *Azotobacter vinelandii,* a species of *Clostridium* isolated from a soybean nodule, and *Alcaligenes radiobacter.* Details of the specific isotopic technics are given by Burris, Eppling, Wahlin, and Wilson.[5] Incubation in the presence of the N^{15} was for 48 hours at 24-29°C.

Results and Discussion. Results from typical experiments are summarized in Table I. The outstanding conclusion is that none

[4] Almon, L., and Baldwin, I. L., *J. Bact.,* 1933, **26**, 229.

[5] Burris, R. H., Eppling, F. J., Wahlin, H. B., and Wilson, P. W., *J. Biol. Chem.,* 1943, **148**, 349.

of the sterile preparations brought about fixation by the rhizobia or by mixtures of rhizobia and radiobacter, a frequent contaminant of nodules. The positive results with *Azotobacter* and *Clostridium* demonstrate that fixation could occur under the experimental conditions. As shown in *Experiment* 5 fixation is not always obtained with *Clostridium* probably because of the presence of free oxygen and soluble organic nitrogen. Neither of these would prevent fixation by *Azotobacter*. To reduce the level of soluble available nitrogen, one experiment was made using a purified hemoprotein solution and also hemoglobin from blood. Neither induced fixation by the rhizobia, nor did the addition of oxalacetate alter this result.

Nonsterile prepartions did not consistently induce fixation, the positive results being concentrated in two experiments. The results with the nonsterile filtrates are significant for the argument that lack of fixation by pure cultures of the rhizobia arises through loss of this physiological function after laboratory cultivation. In these experiments preparations were obtained that, although filtered, contained numerous rhizobia. These were identified by microscopic examination, cultural characteristics, physiological tests in litmus milk and veal infusion broth, and by inoculation of the host plant. Such organisms, tested for fixation immediately after recovery from the nodule and prior to any transfer on artificial medium, were unable to fix even the minute quantity of nitrogen that can be detected by the isotopic method (Experiment 7, 8, and 9). In the positive trials with nonsterile filtrates (Experiment 6 and 10) species of *Clostridium* demonstrated to be capable of fixing nitrogen were isolated.

Summary. When tested by the sensitive isotopic method, neither an aqueous extract of nodules nor a purified preparation of hemoprotein could induce fixation of atmospheric nitrogen by free-living *Rhizobium*.

Cultures of the root nodule bacteria taken directly from the nodules and tested prior to cultivation on laboratory media did not fix nitrogen even though hemoprotein was present.

In a few experiments, fixation of molecular nitrogen was obtained, but the nodular preparations were shown to contain nitrogen-fixing clostridia.

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Effect *in vitro* of Specific Lipid Fractions of Animal Sera on Psittacosis Virus.

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Casals and Olitsky[1] have reported that a number of neurotropic viruses were inactivated when mixed with various animal sera and incubated at 37°C. Furthermore when these sera were successively extracted with acetone, ether, and hot alcohol-ether, each extract was effective in inactivating virus. This report attempts to confirm these results —using psittacosis virus—and to isolate that lipid fraction having the virus-inactivating property.

Method and materials. Virus: Psittacosis virus originally isolated by Dr. Dorland Davis from a fatal disease in a parrot ("4" strain) was propagated in the allantoic sac of 8-day-old chick embryos. Allantoic fluid was

TABLE I.
Fifty Percent End-point of Psittacosis Virus Incubated at 37° in Presence of Serum, Saline and Sterile Water.

	1 hr	2 hr	4 hr	24 hr
Serum	$10^{-6.0}$	$10^{-6.0}$	$10^{-5.8}$	$10^{-3.6}$
Water	$10^{-6.0}$	$10^{-6.0}$	$10^{-4.2}$	$10^{-1.1}$
Saline	$10^{-6.1}$	$10^{-5.8}$	$10^{-2.4}$	10^{-0}

[1] Casals, J., and Olitsky, P. K., *Science*, 1947, **106**, 267.