together with standard biological procedures, indicates that the eggs of the American lobster at the time of attachment to the caudal appendage contain estrogenic activity estimated at a minimum of 100 international units per 100 g of eggs. That a substantially increased yield may be affected by further refinements in extraction methods is now being investigated.

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Microdetermination of Steroid Estrogens in Urine by Fluorometry.*

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We have previously described[1] a fluorometric method for determination of estradiol, estrone and estriol in pure solution, which was much more sensitive and accurate than the colorimetric methods used in determining these substances. The fluorometric method was only slightly less sensitive for estradiol and estrone than the biological tests, while estriol could be determined at levels too low for biological assay. In all cases the accuracy of the fluorometric method exceeded that of the biological method and the range of error was found to be $\pm$ 3%.

The specificity of the new reaction was high, only very few substances interfering with the determination of the estrogens; and since none of these were phenols they could easily be separated from the estrogens and therefore represented no problem in the assay.

The present paper deals with adaptation of the fluorometric method to determination of estradiol, estrone and estriol in urine.

Experimental. (With the assistance of Mrs. B. Wolman.) The following apparatus and reagents were employed in the tests:

1. Coleman Electronic Photofluorometer, Model 12A, with filters B_2, B_1 (optical), PC_2 and PC_9.
2. All-glass distillation apparatus.
3. Test tubes with ground glass stoppers (Pyrex, 15 ccm).
4. Reagents.
   a. Ether, peroxide, free, redistilled.
   b. Sodium bicarbonate, anhydrous, pro anal.
   c. Sodium carbonate, anhydrous, pro anal.
   d. Hydrochloric acid, concentrated, pro anal.
   e. Sulfuric acid, pro anal., diluted with distilled water 4:5.
   f. Sodium hydroxide, pro anal.
   g. Ethanol, redistilled.
   h. Benzene, triophene free.
   i. Phosphoric acid, purest, s.g. 1.75.
   j. Sodium chloride, pure.
   k. Crystalline estrogens.†

A. *Determining the standard estrogen curves.* The Coleman fluorometer provided with the B_2 primary filter and the PC_2 secondary filter was calibrated with 7 cc of a sodium fluoresceinate solution (0.05 γ / cc), so that the value 80 was recovered on the galvanometer scale.

Crystalline estradiol, estrone and estriol dissolved in pure ethanol 96% were used in preparing the standard curves. Substantially smaller amounts of estrogen were used in determining the standard curve than in our previous study,[1] as the response in the Coleman fluorometer was much higher than

* We are deeply indebted to Dr. B. Y. Brent for supplying us with estradiol and estrone, and to Dr. Oliver Kamm for estriol.

1 Finkelstein, M., Hestrin, S., and Koch, W., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 64.

† We are deeply indebted to Dr. B. Y. Brent for supplying us with estradiol and estrone, and to Dr. Oliver Kamm for estriol.

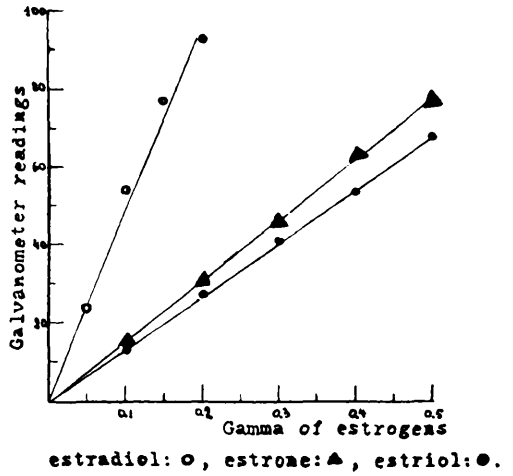

FIG. 1.
The standard curve of estrogens.

that obtained in the Lumetron fluorometer previously employed. The curves of estradiol, estrone and estriol were linear throughout the scale and the limits were 0.05 γ to 0.20 γ for estradiol and 0.1 γ to 0.6 γ for estrone and estriol.

Our procedure in preparing the standard curves was essentially similar to that in our earlier paper, except that 7 cc rather than 2.5 cc of phosphoric acid was used in developing and reading the fluorescence. The standard curves are presented in Fig. 1.

B. *Fractionation of urine in the estradiol-estrone and estriol fraction.* We generally followed the method of Bachmann & Pettit[2] in fractionation though some of the steps in the original method were modified and simplified. Only 10 cc of urine were required in our method, compared with 100 cc in the original method. The recovery percentages, however, fell within the same limits as those described by Bachmann & Pettit.[2] The procedure was as follows:

10 cc of fresh urine were rendered acid with 0.7 cc concentrated HCl (pro anal.) and hydrolyzed under reflux for 1 hour in an all-glass apparatus. The urine was then cooled under running tap water, 5.6 g of sodium chloride was added to it, and it was diluted with 10 cc of distilled water. The solution was extracted 5 times with 20 cc of benzene in a separatory funnel. The pooled benzene extracts (100 cc) were washed in the funnel with 3 cc of sodium bicarbonate 9%, following which the benzene was concentrated to 30 cc. The benzene extract was then shaken in a separatory funnel once with 30 cc and twice with 15 cc of sodium carbonate 9%, and once with 7 cc of water; and the aqueous washings and the sodium carbonate extracts were pooled. Following this procedure all the estradiol and estrone remained in the benzene fraction, while the estriol was almost quantitatively removed in the sodium carbonate fraction. From this point on the two fractions were treated separately.

The estriol fraction. The pooled carbonate extract was rendered acid (less than pH 6) with concentrated HCl and then 3 times extracted with 35 cc of ether. The pooled ether extracts were washed twice in a clean separatory funnel with 10 cc of sodium bicarbonate 9% and twice with 10 cc of water. The ether was then evaporated and the residue was taken up in a measured volume of ethanol, between 10 and 20 cc, and aliquots of this solution were used for fluorometry. In cases when the ether residue was colored, the following additional purification was employed. The residue was taken up in 0.5 cc of ethanol and the solution was diluted with 50 cc of benzene, washed with 2 cc of sodium bicarbonate 9%, and extracted 3 times with half its volume of 0.01 N NaOH and once with 10 cc of water. The pooled extracts were made acid with concentrated HCl and extracted 3 times with half their volume of ether. The ether was evaporated to dryness and the residue was taken up in a measured volume of ethanol, and aliquots of this solution were used for fluorometry.

The estradiol-estrone fraction. The concentrated benzene extract was washed once with a quarter of its volume of dilute sulfuric acid and twice with 15 cc of water. It was then extracted 4 times with an equal volume of N NaOH. The pooled alkaline extracts were acidified with concentrated HCl (pH less than 5) and were then extracted 3 times with 50 cc of ether. The combined

[2] Bachmann, C., and Pettit, D. S., *J. Biol. Chem.*, 1941, **138**, 689.

TABLE I.
Recovery of Estradiol, Estrone or Estriol from 10 cc Urine Samples.

Estrogen added	γ of estrogen added	γ of estrogen observed after subtraction of the blank value	% of recovery
Estradiol	1	0.6	60
"	10	8	80
"	100	84	84
Estrone	50	37	74
"	50	35	70
"	100	80	80
Estriol	100	56	56
"	100	42	42
"	50	28	56
"	50	30.5	61
"	25	12.5	50
"	25	17	68

extracts were concentrated to about 50 cc, washed once with 10 cc of dilute sulfuric acid, twice with 20 cc of sodium carbonate 9% and twice with 20 cc of water. The ether extract was evaporated, the residue was taken up in a measured volume of ethanol, and aliquots of this solution were used for fluorometry.

C. *The fluorometric measurement.* Aliquots in Pyrex test tubes with ground glass stoppers were evaporated to dryness in an electric oven at about 120°C with the stoppers removed. After cooling, 7 cc of phosphoric acid were added to each, the tubes were stoppered and heated in a boiling water bath in the dark for half an hour. When the tubes were cooled, the fluorescence was measured in the Coleman fluorometer, using filters $B_2 + PC_2$. For blank readings the measurement was repeated using filter B_1 (optical) + PC_2, and this value was subtracted from the first readings.

Results. In the recovery experiments the tested estrogen was added to 10 cc of hydrolyzed urine. A blank experiment for this sample was run at the same time, and the value thus obtained was subtracted from the experimental reading. Table I summarizes the results of the recovery experiments.

As may be seen from the table, the best estrogen recoveries (above 80%) were obtained with estradiol. As little as 1 γ of estradiol added to 10 cc of urine could be demonstrated with 60% recovery. The recoveries of estrone were in the range of 70%, and those of estriol were lower, between 50 and 60%. Since an inhibitory effect was observed when concentrated aliquots of the estriol fraction were used, we recommend the use of diluted samples of this estrogen for fluorometric assay.

Discussion. The present study describes an accurate and time-saving method for the determination of urinary estrogens, which is also specific and is not influenced by substances accidentally occurring in the urine. Its particular advantage is its sensitivity, which permits the use of a urine sample as small as 10 cc of normal urine and even less of pregnancy urine. The fluorometric readings are not complicated by interfering substances in the urine, as are the colorimetric readings according to Kober.[3] Thus the estrogen concentration may be calculated in a single reading. Recently, Jailer[4] adapted our fluorometric method to the analysis of estrogens in urine. In a preliminary study he reported the recovery of estrone and estradiol, using diluted sulfuric acid instead of phosphoric acid. This modification, however, proved to be of less value than the original method, since estriol was not detectable by this method, and since his recoveries of estradiol and estrone were in the range of 50%. Furthermore, some of the sterolic substances which did not fluoresce when phos-

[3] Kober, S., *Biochem. J.*, 1938, **32**, 357.

[4] Jailer, W., *Endocrinology*, 1947, **40**, 198.

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.

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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.