free phenol values, not at any time is there present enough free phenol in the blood to produce the symptomatic picture of phenol poisoning or the uremic syndrome. This is contrary to the evidence of Becher[1] and Becher and Koch.[3] The present author, with Becher and Litzner,[4] Becher *et al.*,[13] Dickes,[8] Roen[10] and Nesbit *et al.*,[15] finds that the conjugated and hence the total phenols increase in the uremic syndrome, although the values given in this report are much lower than those of these authors.

Summary. In 14 clinical cases involving uremic syndrome herein reported upon, it was found that:

a) The free phenols had little if any effect in producing or intensifying the uremic syndrome. b) During the uremic syndrome the free, conjugated and total phenols did increase, but not significantly enough to warrant their consideration as an important factor in the uremic syndrome. The methods employed in previous reports give reason to doubt the validity of conclusions drawn concerning the relationship of free, conjugated and total phenols to the uremic syndrome.

16660

Fluorimetric and Biological Determination of Estrogens in the Eggs of the American Lobster (*Homarus americanus*).*†

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The presence of hormones in the tissues and body humors of a variety of invertebrates, both marine and terrestrial, appears to be well-established.[1] Among these, substances having estrogenic potencies are not uncommon.[2] However, satisfactory procedures for producing extracts free of contamination and for testing the potency of such extracts by fluorimetric, as well as the usual biological technics, have only recently become available. This study was made to determine the estrogenic potencies of the eggs of a representative marine invertebrate—the American lobster.

Materials and Methods. A total of 395 g (wet weight), about a quarter of a million eggs, were used, the total yield of 9 specimens. All the eggs were recently spawned and attached to the caudal appendage. The eggs of the lobster remain within the body cavity for nearly a year following ovulation so that the eggs used in this study were all about 11 months old. The extraction procedure follows: The eggs are thoroughly macerated in a Waring Blendor in twice their volume of 95% ethyl alcohol and allowed to stand for 24 hours. The alcohol is filtered off and the residue re-extracted with alcohol for 24 hours. The alcoholic extracts are combined and shaken thoroughly for a half hour with an equal volume of petroleum ether. The ether fraction is removed and the alcoholic fraction is again shaken with ether. Following this, the alcoholic fractions are combined and evaporated to dryness and the deep orange residue is taken up in toluene as a supersaturated solution. For purposes of further purification preceding fluorimetric and biological analysis 5 cc samples were taken of the toluene concentrate. Five cc of the toluene solution are shaken with 10 cc 1N NaOH

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1 Hanström, B., *Hormones in Invertebrates.* Oxford Press, 1939, Chap. 2, p. 22.

2 Donahue, J. K., *Endocrinology,* 1940, **27**, 149.

and slowly centrifuged for 10 minutes. The oily top layer is carefully removed and kept. The NaOH layer is neutralized to pH 3 with concentrated HCl under a small quantity of toluene. The toluene fraction is removed and kept. The oily fraction from the first NaOH separation is treated 5 times in the manner just described and the toluene fractions combined and reduced in volume to about 5 cc. Where the toluene samples appear slightly cloudy owing to the presence of small amounts of oil, another extraction with NaOH may be required.

Fluorimetric Studies. Colorimetric methods for the quantitative determination of estrogens have been greatly improved[3] but where relatively minute amounts of estrogen are involved, fluorimetric analysis appears to be more sensitive.[4,6] For purely qualitative analysis 0.2 to 0.5 cc of the purified toluene extract were placed in a fluorimetric tube and heated with 1 cc 90% sulfuric acid in a water bath at 80°C for 10 minutes. When held up to a narrow beam of light and viewed at an angle of 90° extracts prepared as described showed a blue-green fluorescence easily discernible with the naked eye.

For quantitative work the procedure outlined by Bates and Cohen was followed.[5] Using a Coleman photofluorometer fitted with a B_1 filter at the photocell, subtracting the non-specific fluorescence developed by a blank solution, and comparing the intensity of fluorescence given by the sample with that developed by a standard solution of estrone, it was estimated that each cc of toluene contained between 30 and 50 international units of activity. However, later determinations made in Dr. Bates' laboratory employing a filter combination consisting of a 420 mu Baird Interference Filter at the light source and a combination of a 520 mu Baird Interference Filter and a 3387 Corning Filter at the photocell gave readings of the order of 5 units per cc of toluene. It is believed that this filter combination eliminates virtually all non-specific fluorescence. On the basis of these readings it is estimated that the original 395 grams of eggs yielded between 400 and 500 units of recoverable activity.

Biological Studies. The response of the 150 g spayed rat to injections or vaginal applications of corn oil extracts of the toluene solutions correlated quite closely to the activity as judged by fluorimetric analysis. Ten 150 g rats were injected subcutaneously with the equivalent of 2.5 cc of toluene solution in 3 divided doses over a period of 3 days. The activity was transferred to corn oil by extracting the toluene solution with NaOH and then neutralizing the NaOH to pH 3 under a small quantity of ethyl ether which was mixed with corn oil and allowed to evaporate at room temperature. Ten more spayed females were given direct applications of corn oil solutions to the vagina in the same dosage as above. In taking smears and making vaginal applications great care was taken to avoid trauma. Within the 3-day period all animals showed a clearing of leucocytes and the appearance of large numbers of nucleated epithelial cells. In the injected series 2 animals showed some cornified cells at the end of 3 days. In the intravaginal group 5 of the 10 animals showed large numbers of cornified cells although in no cases were there full estrus responses. Each animal received the equivalent of 1.2 μg of estrone so that, in view of the observation that the rat is some 10 times less sensitive to estrone than the mouse, the dosage given would be close to the threshold necessary for a full response. Until larger quantities of eggs are obtained and highly concentrated extracts prepared from them the consistent production of full estrus smears must wait. In all animals tested, histological study of the reproductive tracts showed varying degrees of growth of the vaginal epithelium up to 13 cell layers of new tissue but relatively slight growth of the uterine epithelium.

Summary. This preliminary study, utilizing recently developed fluorimetric technics

[3] Cohen, H., and Bates, R. W., *J. Clin. Endocrinology*, 1947, **7**, 701.

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

[5] Bates, R. W., and Cohen, H., *Fed. Proc.*, 1947, **6**, 236.

[6] Jailer, J. W., *Endocrinology*, 1947, **41**, 198.

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.

16661

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.

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