mouth. To make a crude estimate of these factors we placed a standard mouse with a muzzle on in the cage with estrone-treated animals. A mouse in a paraffin sack, with only its head exposed, was also placed in a cage with estrone-treated animals. Two other muzzled and sacked animals were placed in the bottoms of cages of estrone-treated animals. The cages have false bottoms. Except for the period of exposure each standard mouse was caged separately. All four of these mice went into persistent estrus, indicating that all four probable routes are effective.

The volume and precision of work on the sex hormones, and on carcinogenic substances will increase and, in some instances, overlap. The transmission of estrogenic substances from animal to animal has not been previously reported. This observation is recorded in the hope that it will prevent such confusion as occurred concerning vitamin B, due to the transmission of the vitamin from animal to animal by excreta. The significance of the transmission of estrogenic substances from animal to animal is: one, that where animals are being treated with large amounts of estrogenic substances they should be kept segregated from any other animals under observation for estrus; and, two, where precision in dosage is required, animals should be kept in individual cages with false bottoms.

9140

Method of Preparing Mice for Quantitative Determination of Urinary Estrogen.*

ALLAN PALMER. (Introduced by C. D. Leake.)

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The methods of biological assay for urinary estrogen in use at the present time are dependent upon the production of estrus in a castrated mouse or rat. Various modifications of the original Allen-Doisy test have been used, such variations being single or multiple injections of either aqueous, glycerol, or oily extracts into castrated or immature normal mice or rats. Methods utilizing male animals and fish have not as yet proved of any great value for quantitative purposes.

Chemical methods for the determination of urinary estrogen have

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been proposed by Kober,[1] Cohen and Marrian,[2] David,[3] Cartland *et al.*,[4] Schmulovitz and Wylie,[5, 6] and Pincus *et al.*[7] Chemical tests based on color changes in the test tube and comparison with standards are preferable to biological tests. The disadvantage of chemical tests is chiefly their inapplicability to the estimation of estrogens in the urine of non-pregnant women. For this purpose we must, for the present, still resort to biological means.

A biological test for urinary estrogen must be for the biological activity of all the estrogenic substances present in the urinary extract. The test cannot be specific for, or differentiate between, different forms of the hormone. The advantage of the biological method is its ability to demonstrate very small amounts of estrogenic hormone in the presence of inactive impurities. The disadvantages are the troublesome time consuming procedures and the necessity of maintaining a supply of test animals. The greatest disadvantage is the source of error and variation in methods. More than a 4000% variation can be demonstrated in the procedures[8] now used.

This paper is presented in an effort to decrease the large percent of error and variation. Our test animal by choice has been the castrated adult female mouse. Just as chemically pure hormone for assay is tested against a known standard hormone, so should urinary extracts for quantitative hormone determination be tested against a standard, or in animals that have been prepared with a known standard hormone preparation.

When daily vaginal smears from large groups of adult virgin mice were examined over a period of two to three weeks, from 30-40% of these animals were found to be having regularly occurring estrus cycles. The animals in such a group were castrated and tested with an estrogen, and it was found that those animals having a cycle reacted more consistently to the minimal effective dose of estrone than those not having a cycle. The method employed at first was to follow a group of animals from day to day from the

[1] Kober, S., *Biochem. Z.*, 1931, **239**, 209.

[2] Cohen, S. L., and Marrian, G. F., *Biochem. J.*, 1934, **28**, 1603.

[3] David, K., *Acta brev. Neerland.*, 1934, **4**, 64.

[4] Cartland, G. F., Meyer, R. K., Miller, L. C., and Rutz, M. H., *J. Biol. Chem.*, 1935, **109**, 213.

[5] Schmulovitz, M. J., and Wylie, H. B., *J. Lab. and Clin. Med.*, 1935, **21**, 210.

[6] Schmulovitz, M. J., and Wylie, H. B., *J. Biol. Chem.*, 1936, **116**, 415.

[7] Pincus, G., Wheeler, G., Young, G., and Zahl, P. A., *J. Biol. Chem.*, 1936, **116**, 253.

[8] MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., *J. Biol. Chem.*, 1936, **115**, 435.

standpoint of their vaginal smears and castrate them as soon as estrus occurred. Almost without exception these animals, so castrated, responded one week later in full estrus to the minimal dose of estrone which we found to be 0.1 gamma. Later it was found to be more time-saving to castrate a group of animals and one week later inject all of them with 0.1 gamma of estrone. It was found that the same percentage (30-40%) of animals responded to the minimal dose and we had excluded the necessity of taking smears beforehand. In a separate experiment it was found that as little as 0.05 gamma estrone would produce estrus, but this was rare and inconstant as compared with the reactivity to 0.1 gamma. If an active animal were castrated and allowed to remain unstimulated for too long a time (10-14 days or longer) it could not be expected to, and will not respond to the minimal or even several times the minimal dose of estrone. Fortunately 7 days has proven to be a good interval for the artificial production of estrus in a castrated animal by the injection of known or unknown amounts of estrogen.

After the animal has been so prepared and tested it is ready for use as a group I animal in testing unknown extracts. Injections of extract are made weekly and the vaginal reaction is followed from day to day. No animal is used for testing an extract oftener than once in 2 weeks. The second week after testing an extract the animal is always given a standard dose whether it has responded to the previous test dose or not. The animals will not always respond to this standard dose again and if not the dose is repeated the following week. *No animal is used for testing again that has not responded in full estrus to a known standard dose one week before.*

The next and most recent step forward has been a plan whereby more than 30-40% of the animals castrated can be made use of in our determinations. We were able to make use of three groups of animals. Group I consisted of those responding to 0.1 gamma, group II those responding to 0.2 gamma, and group IV, those responding to 0.4 gamma estrone. Experimental details are omitted, but the plan evolved is as follows: Each of a group of animals is castrated and one week later given 2.0 gamma estrone in 0.2 cc. olive oil subcutaneously. All the animals react to this overdose, obviating the necessity of taking smears during the second week. On Monday of the third week each animal is injected with 0.4 gamma of estrone. Those responding are given 0.2 gamma the fourth week. Those not responding to 0.4 gamma are discarded. Those responding to 0.4 gamma and not to 0.2 are grouped as IV. Those responding to 0.2 are given 0.1 gamma. Those responding to 0.2 and not to 0.1 gamma are grouped as II, while those responding to 0.1 gamma are

grouped as I. In following the three groups of animals from day to day, one must take into account the same principle of keeping each animal controlled as described above for group I. The group I animals can be used for testing a short while only and soon have to be put into the II group. The latter eventually comes to be the largest as it draws from groups of newly castrated animals as well as from group I. The usefulness of the animals in group IV is also transitory, some of them becoming II's and the others shortly becoming inactive to 0.4 gamma estrone.

The problem of proper variation in the spacing of the amount of urinary extract to be injected presented itself. Interpolated values must be as close together as possible for the sake of accuracy and yet far enough apart so that the inherent variability of the individual animal would not present itself to the extent that two animals receiving a given dose of urinary extract would not react at all, while one or both of the two animals receiving the next smaller dose would go into full estrus. We resorted to trial and error methods and the amounts shown in the accompanying table seem most practical. Two animals are injected with each dose. The test is made for no less

TABLE I.
Injection Table.

Urinary extract (whole)			Urinary extract (1-10 Dil.)			Urinary extract (1-100 Dil.)		
Test dose cc.	*Group*	*Value* γ	*Test dose* cc.	*Group*	*Value* γ	*Test dose* cc.	*Group*	*Value* γ
1.0	I	1.0/L	0.5	II	40.0/L	0.5	II	400.0/L
0.5	I	2.0/L	0.3	II	66.6/L	0.3	II	666.6/L
0.5	II	4.0/L	0.2	II	100.0/L	0.2	II	1000.0/L
0.3	II	6.6/L	0.1	II	200.0/L	0.1	II	2000.0/L
0.2	II	10.0/L	0.1	IV	400.0/L	0.1	IV	4000.0/L
0.1	II	20.0/L	Urine volume for 24 hr.: ——cc.					
0.1	IV	40.0/L	Estrogen assay 24 hr.: ——gamma. (Estrogenic equivalent to estrone)					

than 1.0 gamma per liter of urine since we have never found less in the urine of normal adult women. Extracts are 100-fold concentrates of the urine. Their preparation is by benzene extraction, combining the method of Cohen and Marrian[9] for maximal recovery with minimal destruction of hormone and a suggestion from Schmulovitz and Wylie[10] which makes use of the protective and washing properties of sodium carbonate.

Daily examination of smears, for 2 to 4 months from several

[9] Cohen, S. L., and Marrian, G. F., *Biochem. J.*, 1935, **29**, 1577.

[10] Schmulovitz, M. J., and Wylie, H. B., *Mimeographed Supplement to reference No. 5.*

hundred castrated animals over a period of 2 years, has indicated that the necessary smears for practical use should be 48, 56, 72, 80, and 96 hours after the animal receives its injection. If the animals are injected on Monday A. M. this arrangement calls for smears to be recorded Wednesday A. M. and P. M., Thursday A. M. and P. M., and Friday A. M. If a full estrus reaction is not found during these intervals the result is negative. When estrone is administered the smears taken at the same intervals should also reveal a full estrus reaction or the animal should not be considered controlled. The route of injection must be subcutaneous, both with the standard and the unknown; injections must be single in number and always in the same solvent, olive oil. The use of fewer than 20 animals as stipulated for each test dose in the Report of the Second Conference on the Standardization of Sex Hormones[11] can be satisfactory only if these animals are individually examined and suitably controlled.

These studies do not indicate that different groups of mice have a definite constant threshold of reactivity to estrone but rather that by first overstimulation and then persistent adequate stimulation at regular intervals mice can be brought into, and then maintained in, a state of maximal reactivity to a rather constant minimal dose of estrone.

9141 P

Distribution of Phosphorus in the Starch Granule.

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Gelatinized root-starch granules possess a peripheral membrane containing phosphoric-acid ester. This has led some investigators to infer that the natural ungelatinized granule also possesses such a membrane. If this be so, it follows that the P_2O_5 percentage in the small granules of a given sample should be greater than in the large granules, provided the thickness of the hypothetical membrane of the two sorts of granules is about the same.

There are two pertinent observations in the literature. Fernbach[1] found the small-sized granules to contain decidedly more P_2O_5 than

[11] *Quart. Bull. Health Organization League Nations*, 1935, **4**, 618.

[1] Fernbach, A., *Compt. rend.*, 1904, **138**, 428.