It seems, therefore, that the tannic-acid treatment, which is believed to act principally on the mucosa[1] of the nose, does not influence in any way the course of at least one intranasal bacterial infection. It should, however, be emphasized that pneumococci are believed to invade the body also through the pulmonary alveoli after intranasal instillation.[4]

8682 P

Concentration and Purification of the Gonadotropic Substance in Urine of Ovariectomized and Post-Menopausal Women.*

LOUIS LEVIN† AND H. H. TYNDALE. (Introduced by P. E. Smith.)

From the Department of Anatomy, College of Physicians and Surgeons, Columbia University.

While attempting to concentrate the gonadotropic activity of the urine of post-menopausal (or castrate) women, it has been found that the active material may be practically quantitatively concentrated into relatively non-toxic extracts by the use of tannic acid. The method developed for the precipitation and partial purification of the gonadotropic material is as follows.

The chloroform-preserved urine is chilled, siphoned from any sediment and acidified to pH 5 with acetic acid. For each liter of urine, 20 cc. of a freshly prepared aqueous 10% tannic acid solution are added. An immediate precipitate forms. After thorough mixing, the precipitate is allowed to settle in the cold room and is then collected at the centrifuge. The bulky precipitate, which contains considerable water, is extracted once with at least 5 parts of 95% ethyl alcohol followed by 3-4 extractions with 80% alcohol. Alcohol is removed by several washings with acetone and the residue is freed of acetone by reduced pressure. In addition to removing considerable pigment and other inert material, the alcohol-acetone treatment removes any estrogenic substances which may be present.

The resultant powder (100 to 200 mg. per liter of urine) con-

* A portion of this work was reported before the American Society of Biological Chemists at Detroit, April 13, 1935 (*J. Biol. Chem. (Proc.)*, 1935, **109**, liv.)

Aided by a grant, administered by Dr. P. E. Smith, from the Committee for Research in Sex, the National Research Council.

† Squibb Fellow, 1935-36.

tains 75-100% of the original gonadotropic activity of the urine‡ as indicated by parallel assays of the raw urine (or alcohol precipitates thereof). The activity of the dry tannate is quite stable, no loss being noted even after storage for more than a year at room temperature.

Assays are conducted by giving 20-22-day-old mice (8-12 gm. body weight) subcutaneous injections once daily for 3 days, the animals being sacrificed 72 hours after the first injection. Because with minimal doses ovarian weight alone is an unreliable criterion, the mouse unit has been tentatively defined as that amount which, when administered in the above manner, will cause vaginal canalization, at least a 200% increase in the weight of the uterus drained of fluid (controls 4-7 mg.), and a slight increase in ovarian weight as compared to uninjected controls. Such a unit is very sensitive and in the absence of estrogenic substances is quite accurate. We have never been able to demonstrate the presence of any estrogenic activity in the alcohol washed tannates.

Partial purification of the crude tannate is accomplished as follows. The dry tannate is thoroughly extracted 4-5 times with a barium hydroxide-barium acetate mixture at pH 9.0-10.0. Each extract is immediately adjusted to pH 7.0-8.0 with dilute H_2SO_4 and the combined solutions are freed of excess barium by addition of $(NH_4)_2SO_4$ solution, dilute ammonia being added, if necessary, to maintain the pH at 7.0-8.0. The barium sulphate is collected and leached several times with small volumes of dilute NH_4OH. The combined extract and washings are chilled, adjusted to pH 9.0-10.0 by cautious addition of ammonia water and immediately precipitated by the addition of 2-3 volumes of cold acetone. After chilling over night, the precipitate is collected at the centrifuge, washed with acetone and freed of acetone by reduced pressure. The product is an almost white powder which is completely soluble in water. Such powders retain 70 to 100% of the activity of the original tannate but only 15 to 25% of the original solids.

‡ Application of this precipitation to other types of urine (pregnancy, normal women, normal men) has yielded excellent results. Hellbaum *et al.*[1] have reported similar experience with pregnancy urine. Others[2, 3, 4] have applied our method to normal urines with confirmatory results.

1 Hellbaum, A. A., Fevold, H. L. and Hisaw, F. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1566.

2 Thomsen, O., and Pedersen-Bjergaard, K., *Compt. rend. Soc. d. Biol.*, 1935, **120**, 1143.

3 Pedersen-Bjergaard, K., *Zentralbl. f. Gynak.*, 1936, **60**, 372.

4 Friedman, M. H., and Weinstein, G., *Proc. Am. Physiol. Soc.*, 1936, p. 54.

More than half of the remaining inert material may be eliminated, with little or no loss of activity, by the following procedure. The powder is dissolved in 60% ethyl alcohol at pH 9.0-10.0 (ammonia water drop by drop). The somewhat turbid solution is then brought to pH 6.5 by the dropwise addition of glacial acetic acid. The precipitate which forms is immediately removed by centrifugation and is washed several times with small volumes of slightly acidified 60% alcohol. The clear brown alcoholic solution and washings are combined and an equal volume of acetone is added. The mixture is chilled over night, the precipitate collected, washed with acetone and freed of acetone by reduced pressure. The product is a white powder (10-15 mg. from each liter of pooled urine), readily and completely soluble in water and active in mice in total doses of 0.10-0.15 mg. Solutions in which the original activity of the urine is concentrated as much as 10-20 thousand times are easily prepared.

This material produces no toxic effects even when administered in very large doses. Hypophysectomized immature rats, which are extremely sensitive to toxins, have shown no ill effects when injected with as much as 1,000 mouse units (equivalent to 5-10 liters pooled menopause urine) over a period of five days.

These preparations contain no tannic acid but are completely precipitated from acidified solution by the addition of tannic acid. However, no precipitation is obtained with the other alkaloidal reagents except phosphotungstic and phosphomolybdic acids. This may explain why tannic acid is much more efficient than are the other protein precipitants in the initial precipitation of the activity from the urine.