8916 P

Observations on the Conjugated Oestrogens in the Urine of Pregnant Mares.

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Recently in this laboratory it was shown[1, 2] that during the last few days of pregnancy in women, immediately before and during labor, there occurs a rapid fall in the total amount of oestrogen excreted, simultaneously with a very great increase in the ratio of "free" ether-soluble oestrogen to conjugated ether-insoluble oestrogen. It was tentatively suggested that this conversion of the less physiologically potent conjugated hormones to the free hormones might be a factor in the initiation of labor. In order to obtain further evidence to support this theory, attempts were made to carry out similar investigations on other species, so that the research could be extended along more experimental lines. In this paper we report the results of colorimetric determinations[3] of the free and conjugated oestrogen in the urine of several mares at different stages of pregnancy. Previous work from this laboratory and elsewhere[4, 5, 6] has shown that the colorimetric procedure is applicable to pregnant mares' urine.

The estimations were carried out as follows: 100 cc. of fresh urine were adjusted to pH 6.0 after saturation with NaCl, and extracted several times with toluene. The combined toluene extracts were washed with saturated aqueous Na_2CO_3 and then extracted repeatedly with N. NaOH. The NaOH extract was made faintly acid with HCl, then alkaline with Na_2CO_3 and finally extracted with ether. The ethereal extract after washing with water was evaporated to dryness and the "free" oestrogen estimated colorimetrically in an aliquot portion of the residue. The oestrogen present in such a fraction is presumably a mixture of oestrone, equilin, hippulin, equilenin and oestradiol. At the present time no methods for the quantitative separation of these oestrogens from one another

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[1] Cohen, S. L., Marrian, G. F., and Watson, M., *Lancet*, 1935, **1**, 674.

[2] Marrian, G. F., Cohen, S. L., and Watson, M., *J. Biol. Chem.*, 1935, **109**, lix.

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

[4] Beall, D., and Marrian, G. F., *J. Soc. Chem. Ind.*, 1934.

[5] Beall, D., and Edson, M., *Biochem. J.*, 1936, **30**, 577.

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

are available and few data are available on their relative chromogenic powers. Of necessity, therefore, the colorimetric readings have been evaluated in terms of mg. of oestrone. Since the latter is the chief constituent of the mixture of oestrogens in mares' urine, this procedure is considered to be justifiable.

Attempts were made to determine the total oestrogen (free + conjugated) by carrying out a similar procedure on a second sample of urine which had been allowed to stand at pH 1.0 at room temperature for 7 days. We have since had reason to suspect, however, that these conditions are not sufficiently drastic to hydrolyse the conjugated oestrogen completely, and it is doubtful if our figures for the total oestrogen have much quantitative significance. The figures for the conjugated oestrogen showed that the average excretion at about the seventh month was close to 10 mg. (as oestrone) per 100 cc. urine. Wide variations from day to day even in the same mare were observed however. It seems probable that these variations are largely due to the unsatisfactory conditions of hydrolysis of the urine which were employed. From the seventh month onward, the oestrogen content of the urine fell irregularly to about 1-3 mg. per 100 cc. at term. A similar decrease in oestrogen content of the urine during the latter half of pregnancy has been previously reported by other authors.[5, 7, 8] We have not been successful in demonstrating a sudden fall in oestrogen excretion immediately before parturition. However, it is probable that if such a fall did occur, it would have been obscured by the wide day to day variations.

The figures for the "free" oestrogen generally varied between 0.2 and 0.5 mg. per 100 cc. although occasional variations from 0.04 to 1.35 mg. were encountered. These occasional high figures are possibly due to slight bacterial decomposition of the urine. There was no evidence of any regular progressive change in the amount of "free" oestrogen excreted during the last few months of pregnancy. In several instances we examined urine samples obtained from mares the day before and the day after foaling. The figures obtained for "free" oestrogen did not in any instance vary widely from the average excretion during the preceding few months.

We conclude from these experiments that parturition in the mare is not accompanied by marked changes in the ratio of "free' to conjugated oestrogen such as are observed in the human. If qualitatively similar changes do occur they are too small to be detected by the methods of estimation which we have employed.

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[7] Cole, H. H., and Saunders, F. J., *Endocrin.*, 1935, **19**, 199.

[8] Kober, S., *Klin. Woch.*, 1935, **14**, 381.

Our attempts to isolate the conjugated oestrogens in mare's urine have not so far been successful, but since we have obtained evidence on their probable chemical nature, the opportunity is taken to present a preliminary report on the work.

Distribution between various immiscible solvents of an alkali-washed butanol extract of urine from a mare at the seventh month of pregnancy, yielded a white amorphous solid containing about 40% of chromogenic oestrogen (calculated as oestrone). This material was insoluble in ether but easily soluble in water. It gave a negative naphthoresorcinol test indicating the absence of glucuronic acid. Sulphur was present however, and since after hydrolysis with dilute HCl a positive test for inorganic SO_4 was obtained it is possible that the oestrogens are conjugated with sulphuric acid. Millon's test was negative, indicating the blocking of the phenolic hydroxyl of the oestrogen by the conjugating group. Since the preparations obtained were obviously impure, a final decision concerning the chemical nature of these conjugated oestrogens must be deferred.

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8917 P

Determination of Reduced Ascorbic Acid in Blood.

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Since the appearance of the clinical methods for determining blood ascorbic acid by Farmer and Abt[1, 2] considerable interest has been aroused in the study of vitamin C in various diseased conditions. The determination depends on the following proceduure: Deproteinization of the plasma by 10% metaphoric acid[3] and titra-

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[1] Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1625.

[2] Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 146.

[3] Fujita, A., and Iwatake, D., *Biochem. Z.*, 1935, **277**, 293.