FURTHER OBSERVATION OF THE IMMUNOLOGIC DETERMINATION FOR HUMAN LUTEINIZING HORMONE

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SYNOPSIS

1) Extracting 24 hrs. urine by the kaolin-acetone method, immunologic and biologic assays were performed simultaneously for comparative study of luteinizing hormone (LH). Continuous daily measurements in normally menstruating women found the LH peak on time of ovulation; and an approximate agreement was observed between the immunologic and biologic assay. 2) When measuring LH by the hemagglutination inhibition technique, the end point is occasionally difficult to identify because the ring pattern appearing on the bottom of the test tube is atypical. We found that the use of Sephadex G 25 made every ring pattern distinct, almost completely eliminated the atypical ring, and enabled more accurate measuring than without the use of Sephadex. 3) Serial LH variations during one menstrual cycle of each subject showed a fairly good agreement with biologic assay. Immunologic and biologic assays in each urine collected at random showed comparatively parallel results in both assays. However, some cases showed lower values by immunologic than by biologic assay. The possible reasons inducing such dissociation in both assays are discussed.

In 1962, Wide and Gemzell employed the daily urinary immunologic assays for luteinizing hormone (LH) on 3 normally menstruating women and observed a peak of LH excretion coinciding with ovulatory phase (Wide and Gemzell, 1962). Similar findings to the above were later obtained by Sato et al. (1965), Bermes et al. (1966), Mishell (1966), and Miyata (1967).

LH bioassay which proved to be difficult and time consuming has not been used widely for routine clinical investigations. This is true of the test described by Parlow (1958) which depends on ascorbic acid depletion of the ovary and of the test described by McArthur (1958) which estimates the ventral prostate weight of hypophysectomized immature male rats. The immunologic method, however, has some problems which have to be solved by further studies.

In the present study, urinary LH was assayed by the hemagglutination inhibition technique, extracting from 24 hrs.' urine by the kaolin-acetone method. The presence of atypical ring, one of the problems associated with the above-mentioned immunologic procedure, was successfully removed almost completely by means of Sephadex G 25. The immunologic assay was compared with bioassay for determination of the urinary LH levels in normally menstruating women and amenorrheic patients.

MATERIALS AND METHODS

Twenty-four hours’ urines were collected from
5 cycles of 5 normally menstruating women aged from 26 to 36, and from 22 samples of 14 amenorrheic patients aged from 20 to 36. In the former cases all had normal menstrual histories and normal bi-phasic basal body temperature (BBT) records.

Urine extraction: Each urine specimen was extracted by the Igarashi et al.'s (1967) modification of the kaolin-aceton method described by Borth et al. (1961). The extract from the 24 hrs.' urine was dried and then dissolved, as a rule, in 24 ml of isotonic saline.

Immunooassay procedures: 2.5 ml of the solution was mixed well with 200 mg Sephadex G 25 fine*. The mixture was stood for 5 mins., and filtered with Whatman No. 50 filterpaper. The filtered supernatant was used for immunologic determination. The immunologic methods employed were described in detail in our previous publication (Sato et al., 1965). One-quarter milliliter of the filtered supernatant was used for serial dilution with 0.25 ml of isotonic saline; dilutions of 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 were made. The final volume in each tube was 0.25 ml. Human chorionic gonadotrophin (HCG)** standard solution having a concentration of 5,000 I.U. per liter was prepared and diluted serially in the same way as the supernatant samples. HCG-antiserum* (0.25 ml) was added to each tube used and followed by a drop of HCG-sensitized blood cells*. The contents of the tubes were mixed well and kept undisturbed at room temperature. After 2 hrs., the reaction was read on the bottom of each tube. The end point was the highest dilution of an unknown sample which gave the hemagglutination inhibition reaction similar to that obtained as the end point of the standard dilution. If a partial agglutination occurred either in the standard or in the unknown dilution, the end point of the unknown was extrapolated. The concentration of HCG in the unknown sample expressed in international units per liter equaled the concentration of HCG standard, measured in the same units at the end point. Then, the final values were calculated as international units per 24 hrs.

Biological assay: LH of the urinary extract was biologically assayed by Yokota’s modification (1965) of the ovarian ascorbic acid depletion (OAAD) method, in a 4-point design using the standard assayed against NIH-LH-SI***. And in these there was no significant deviation from parallelism between the standard and unknown dose-responses. The results of the

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** The human chorionic gonadotrophin was supplied as Primogonyl by Schering A.G. Company, Berlin, Germany.
*** Obtained from the Endocrinology Study Section, National Institutes of Health, U.S. Public Service.

Fig. 1. Urinary excretion of LH by immunologic (above) and biologic (below) assays and BBT record in Case 1.
bioassays were expressed as \( \mu g \) equivalents NIH-LH-SI per 24 hr. urine.

**RESULTS**

The possible decrease of LH activity under our method using Sephadex G 25 was examined by the OAAD method. The results gave an OAAD of 50.05% for the control and 42.99% after the use of Sephadex, thus with no difference of statistical significance. No significant change in LH activity due to the use of Sephadex was observed.

Data were obtained from 5 cycles of 5 normally ovulating women. In Case 1, the peak of LH was observed on the 14th day of the cycle by immunoassay and on the 13th day by the biological assay, whereas the distinct rise of BBT appeared on the 15th day (Fig. 1). In Case 2, the peak of LH was on day 17 and on day 15 by immunoassay and bioassay, respectively. Significant elevation of BBT was observed on day 19 (Fig. 2). Case 3 showed the peak of LH on the 14th day by both immunoassay and bioassay, whereas the BBT began to rise on the 16th day (Fig. 3). Case 4 whose cycle was irregular showed ovulation with distinct BBT rise on the 32 day. Whereas, there appeared to be two LH peaks by immunoassay, one on day 2 to 3 and another on day 32. The first peak was presumably caused by the mixed presence of much menstrual blood in the urine (Fig. 4). In Case 5, carried on immunoassay alone, the LH peak preceded the BBT rise by one day (Fig. 5). Compar-
ative study of both assays were performed on 22 samples from 14 amenorrheic women (Fig. 6). Parallelism was observed on the whole, though in occasional cases low immunologic values were observed along with high values of bioassay.

**DISCUSSION**

In 1961 Wide, Roos, and Gemzell found that the reaction between HCG-coated cells and rabbit anti-HCG sera was inhibited by concentrates of human hypophyses or of urine from non-pregnant women, pro-
vided they contained LH. They also assayed several human urinary and pituitary concentrates for LH by both the immunologic method and biologic assay, using the rat ventral prostate method. They found that the assay results with the two methods were nearly identical in each substance tested. The mean index of discrimination was 1.04. Those results were later confirmed by Butt et al. (1964) using selected anti-HCG sera, and by Goss and Taymor (1962) using latex-HCG agglutination inhibition. Paul and Ross (1964) employing adsorption studies along with gel diffusion, showed biological as well as immunologic cross-reaction between HCG and LH. McArthur et al. (1958) and Taymor (1961) used the repair of ventral prostatic atrophy of hypophysectomized immature male rats for the determination of LH, and observed that the midcycle peak of LH was closely related to the BBT shift. Using the ovarian-ascorbic-acid-depletion method, Fukushima et al. (1964) demonstrated that the rise of LH excretion preceded the BBT shift by 1—3 days. Those findings are in agreement with the values obtained by Wide and Gemzell (1962), Sato et al. (1965), and Mishell (1966).

By the method we reported earlier (1965), the LH peak either coincided with or preceded the BBT shift by 1 to 2 days, and the LH levels were fairly higher than those reported by Wide and Gemzell (1962). This may have meant the possible mixed presence of some other urinary protein; and 24 hrs. value could not be obtained.

In our present study, we used 24 hrs.' urine which was extracted by the kaolin-acetone method and assayed. The LH peak either coincided with or preceded the BBT shift by 1 to 2 days, agreeing with the peak of bioassay, though with a difference of several days. Tamada (1968), however, found the alcohol-acetone method gave several times higher value than the kaolin-acetone method did.

The problem posed by immunoassay, particularly by the hemagglutination inhibition test, is the shape of the end point ring. A nonspecific ring appears in many cases, making the judgement of the end point difficult. Besides, since serial dilution has been performed, the higher the multiple of dilution, the higher the numerical value of result. The result differs tremendously, for instance, between when the end point is taken at 1/64 and when taken at 1/128. Use of Sephadex G 25 made every ring distinct, almost completely succeeding in doing away with atypical ring. This made it easier to judge the end point and to get more precise values of calculation. Comparing the use and non-use of Sephadex, no difference in the biological activity of LH was demonstrated.

The serial variations of LH during one menstrual cycle in each women were found fairly well in agreement between the immunoassay and bioassay, as mentioned above. Although simultaneous immunoassay and bioassay in urine collected at random in amenorrhoeic patients showed both comparatively in parallel, in some cases immunoassay showed a lower value while bioassay gave a higher value. This seems to result from some essential differences between bioassay and immunoassay. It might be possible that complete difference in the chemical structure of active groups quantitated by the two assays as demonstrated recently by Barr and Collee (1967), or slight but essential difference in the antigenicity of HCG and LH or possible concomitant presence of nonspecific antibodies because HCG used as antigen is not pure enough. Moreover, they may presumably be the interference with erythrocyte agglutination inhibitory reaction by other proteins or other substances rather than gonadotrophin which has been extracted incidentally in the process of gonadotrophin extraction from urine.

LH was found fairly increased when measured during menstrual period. This seems to have raised the value of other protein than LH, because menstrual blood was present mixed in the urine.

LH determination is not only important for further basic understanding of gynecologic
endocrinology, but also necessary for clinicians for diagnosis and treatment of certain types of endocrine diseases. Swift and simple determination of LH is desirable. If and when a fully purified LH becomes available for red-cell coating and for preparation of antisera, immunoassay more precise than practised at present will become feasible. At the present time it will be necessary to continue improving the LH immunoassay which has various problems.

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REFERENCES