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Abstract

An ultrafiltration method employing a Centrifree filter for determining the unbound fraction of estradiol was studied. Centrifugation was performed under conditions similar to those in vivo. Good correlation was recognized between this method and the equilibrium dialysis. This method was employed to determine the unbound fraction of estradiol in the serum and the peritoneal fluid of 26 infertility patients classified according to their menstrual dates. The total estradiol and progesterone contents in the peritoneal fluid were high after ovulation. There was no significant difference in the percentage of unbound estradiol in the serum among various groups. In the peritoneal fluid, however, the percentage of unbound estradiol for the day 12-14 patients was 4.5 +/- 0.2% in contrast with 3.8 +/- 0.4% for the day 15-18 group (p less than 0.05) and 3.5 +/- 0.1% (p less than 0.05) for the day 19-28 group. Moreover, the fraction (4.5%) of unbound estradiol in the peritoneal fluid of a patient with luteinized unruptured follicle (LUF) syndrome was comparable with that of patients in the follicular phase. The difference between the percentage of unbound estradiol in the peritoneal fluid before and after ovulation is considered to be due to the transudation of follicular estradiol in the follicular phase and the exudation of estradiol from the corpus luteum into the peritoneal cavity in the luteal phase.

KEYWORDS: ultrafiltration, fraction of unbound estradiol, luteinizied unruptured follicle(LUF) syndrome, peritoneal fluid

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Determination by Ultrafiltration of the Fraction of Unbound Estradiol and Its Variation in Peritoneal Fluid during the Menstrual Cycle

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An ultrafiltration method employing a Centrifree® filter for determining the unbound fraction of estradiol was studied. Centrifugation was performed under conditions similar to those *in vivo*. Good correlation was recognized between this method and the equilibrium dialysis. This method was employed to determine the unbound fraction of estradiol in the serum and the peritoneal fluid of 26 infertility patients classified according to their menstrual dates. The total estradiol and progesterone contents in the peritoneal fluid were high after ovulation. There was no significant difference in the percentage of unbound estradiol in the serum among various groups. In the peritoneal fluid, however, the percentage of unbound estradiol for the day 12-14 patients was $4.5 \pm 0.2\%$ in contrast with $3.8 \pm 0.4\%$ for the day 15-18 group ($p < 0.05$) and $3.5 \pm 0.1\%$ ($p < 0.05$) for the day 19-28 group. Moreover, the fraction (4.5%) of unbound estradiol in the peritoneal fluid of a patient with luteinized unruptured follicle (LUF) syndrome was comparable with that of patients in the follicular phase. The difference between the percentage of unbound estradiol in the peritoneal fluid before and after ovulation is considered to be due to the transudation of follicular estradiol in the follicular phase and the exudation of estradiol from the corpus luteum into the peritoneal cavity in the luteal phase.

Key words : ultrafiltration, fraction of unbound estradiol, luteinizied unruptured follicle (LUF) syndrome, peritoneal fluid

Peritoneal fluid (PF) has long been known to be present in normal women (1). Variation in the volume and steroid content have already been noticed (2-6). Although its physiological significance is still unclear, the abrupt increase in the volume and steroid content immediately after ovulation may play an important role in tubal transport, fertilization and implantation, and has been paid more attention recently (7).

Protein-bound hormones are not available in target organs and protein-unbound hormones are considered to be the biologically active form. Several methods based upon equilibrium dialysis for determining the unbound hormones have already been reported (8-11), and each has its shortcomings, such as time consuming or meticulous procedures, or requirement of a large sample. Theoretically, ultrafiltration should be equal to equilibrium dialysis for determing the unbound fraction of hormones, and it has the advantage of being simpler to perform (12).

A disposable ultrafilter devised for separating the protein unbound components was investigated for determining the unbound fraction of estradiol, and attempts to carry out the determination under conditions similar to those *in vivo* were performed. This method was then employed to determine the unbound

fraction of estradiol in the serum and the peritoneal fluid of patients.

Because the unbound estradiol concentration in the serum and the peritoneal fluid is usually too low to be measured directly by present radioimmunoassay methods, an isotope labelled tracer was employed to determine the unbound fraction of estradiol.

Materials and Methods

Evaluation of the conditions of the ultrafiltration method. The experiment was carried out using pooled serum prepared by mixing the sera of normal menstruating women, and stored at −20°C.

Apparatus for ultrafiltration. A Centrifree filter® (MPS-3, Amicon Corporation) containing a YMT membrane devised for separating protein bound and unbound components was utilized for separating the unbound estradiol.

Isotope labelled tracer. 3, 4, 6, 7-^{3}H-Estradiol (specific activity 93.2 Ci/mmol) was purchased from New England Nuclear Corporation and purified by column chromatography with LH-20 (Pharmacia Fine Chemicals) immediately prior to use.

Measurement of the fraction of unbound estradiol. Tritium labelled estradiol at the ratio of 0.48 pmol (100,000 dpm) per ml of serum was pipetted into a test tube and evaporated. After pre-equilibration with the serum and buffer, 50 μl of the serum was removed for radioactivity determination (original dpm) and the remainder was transferred to the filter and centrifuged at 1,100×g for 10 min in an angle rotor centrifuge. Fifty μl of the ultrafiltrate was removed for radioactivity determination. The fraction of unbound estradiol was then calculated and represented by the percentage of the total estradiol using the following equation:

$$\%\ \text{of unbound estradiol} = \frac{\text{dpm of the ultrafiltrate}}{\text{original dpm}} \times 100 \qquad (1)$$

Evaluation of the influence of the sample volume. The influence of the volume of the sample for ultrafiltration was studied by putting 0.3, 0.5, 0.7 and 0.9 ml of the equilibrated serum into the filter. The percentage of the unbound estradiol was determined as mentioned above.

Evaluation of the effect of centrifugation time. The serum was applied to the filter and centrifuged at 37°C for 5, 10, 15, 20, 25 and 30 min respectively. The percentage of the unbound estradiol was then determined as mentioned above.

Evaluation of the effect of the temperature. The pooled serum was added to a test tube as described above. Then 0.5 ml of the serum was applied to the filter which was then incubated and centrifuged at 10, 18, 25 and 37°C. The percentage of the unbound estradiol was then determined as mentioned above.

Evaluation of the effect of pH. One molar HEPES buffer adjusted to pH 6.0, 6.8, 7.4, and 8.4 was added to the pooled serum at a concentration of 5% of the volume of the serum. The mixture was then incubated at 37°C for 30 min. The percentage of unbound estradiol at each pH was then determined as mentioned above.

Evaluation of the effect of dilution. The pooled serum was diluted with the HEPES buffer, and the percentage of unbound estradiol at various dilutions was determined. The percentage obtained by equation (1) was corrected for mass action with the dilution factor according to Vlahos (13) by the following equation:

$$\text{Corrected \% of unbound estradiol} = \frac{\text{\% of unbound estradiol}}{\text{dilution factor}} \qquad (2)$$

where

$$\text{Dilution factor} = \frac{\text{serum volume} + \text{buffer volume}}{\text{serum volume}}$$

Standard procedures for determining the fraction of unbound estradiol by the ultrafiltration method. Practical determination of the fraction of unbound estradiol according to the ultrafiltration method was performed as described below. After evaporating 0.29 pmol (60,000 dpm) of the purified tracer in a test tube, 0.6 ml of serum and 30 μl of HEPES buffer (1 mol/l, pH 7.4) were added to the test tube and allowed to equilibrate at 37°C for 30 min. Then, 50 μl of the mixture was removed for radioactivity determination. Five-tenths ml of the mixture was transferred to a filter prewarmed to 37°C and centrifuged at 37°C, 1,100×g for 10 min in an angle rotor centrifuge.

Fifty μl of the ultrafiltrate was then removed for radioactivity determination. The fraction of the unbound estradiol was then calculated with equation (1) and corrected with the dilution factor (1.05) by equation (2).

Comparison with equilibrium dialysis. The ultrafiltration method was compared with equilibrium dialysis following the method of Kurz (14). Equilibrium dialysis was carried out by dialyzing 8 samples of serum in cellophane tubes (Union Carbide) against equal volumes of HEPES buffer (80 mmol/l, pH 7.4) at 60 agitations per min in a 37°C water bath for 18 h. An aliquot (50 μl) of the dialysate was removed for counting. The serum inside the cellophane tube was then immediately transferred to prewarmed filters and ultrafiltered at 37°C as above. The ratio of the count of the ultrafiltrate against that of the dialysate was calculated.

Direct comparison of the unbound estradiol concentrations determined by ultrafiltration and eqilibrium dialysis for 14 serum samples was made as described below. Equilibrium dialysis was performed by dialyzing the undiluted serum against 5 volumes of HEPES buffer (80 mmol/l, pH 7.4) modified after Wu (8), and the percent and concentration of unbound estradiol were calculated by the following equations:

$$\%\ \text{of unbound estradiol} = \frac{\text{dpm of the dialysate}}{\text{dpm of the dialyzed serum}} \times 100 \qquad (3)$$

$$\text{Unbound } E_2\ (\text{pg/ml}) = \text{serum } E_2\ (\text{pg/ml}) \times \%\ \text{unbound} \times 0.01 \qquad (4)$$

Patients. Twenty-six patients observed during the period from May, 1984 to October, 1985 were included in this study. The patients had normal serum blood protein levels, with dating of the menstrual phase possible by basal body temperature recording or endometrial biopsy, and with clear ovarian findings and adequate peritoneal fluid collection possible during the laparoscopic infertility examination.

The laparoscopy was performed under general anesthesia with the patients in the lithotomy position. Peritoneal fluid was aspirated from the cul-de-sac while blood was drawn from the antecubital vein of the patient directly before the operation. Peritoneal fluid with gross blood contamination was excluded from this study. Both the blood and peritoneal fluid were centrifuged, and the supernatants were stored at −20°C.

Endometrial dating was judged according to the criteria of Noyes (15).

Luteinized unruptured follicle (LUF) syndrome was diagnosed by serial ultrasonographic examination and the persistence of the follicles by laparoscopy with evidence of luteinization of the follicle (16-18).

Determination of the fraction of unbound estradiol in the serum and peritoneal fluid was carried out following the standard procedure of the ultrafiltration method described above. The calculation was done using the equations (1), (2) and (4).

Radioactivity determination. The aliquot for counting was pipetted into a minivial (Milli-3, Lummac Co.). After adding 2 ml of Scintisol^{R} (Dotite Co.), the radioactivity was determined for 10 min with an Aloka LSC-700 liquid scintillation system.

Hormonal assays. Estradiol was determined by radioimmunoassay after extraction and chromatography following the method of Yoshida *et al.* (19) with antiserum supplied by Teikoku Hormone Mfg., Ltd..

Progesterone was determined by radioimmunoassay with a progesterone assay kit supplied by Daiichi Radioisotope Labs., Ltd.

Statistical analysis. Significance was evaluated by Student's t test and linear regression analysis.

Results

Changing the volumes of the ultrafiltration samples did not significantly influence the percentage of unbound estradiol. Although the volumes of the ultrafiltrates varied with the volumes centrifuged, the radioactivity recovered in the ultrafiltrate remained constant. The fraction of unbound estradiol calculated did not differ with varying lengths of centrifugation (Fig. 1). The protein concentration of the sample within 30 min of centrifugation did not cause significant variation in the unbound fraction.

Fig. 2 shows the change in the percentage of unbound estradiol at different tempera-

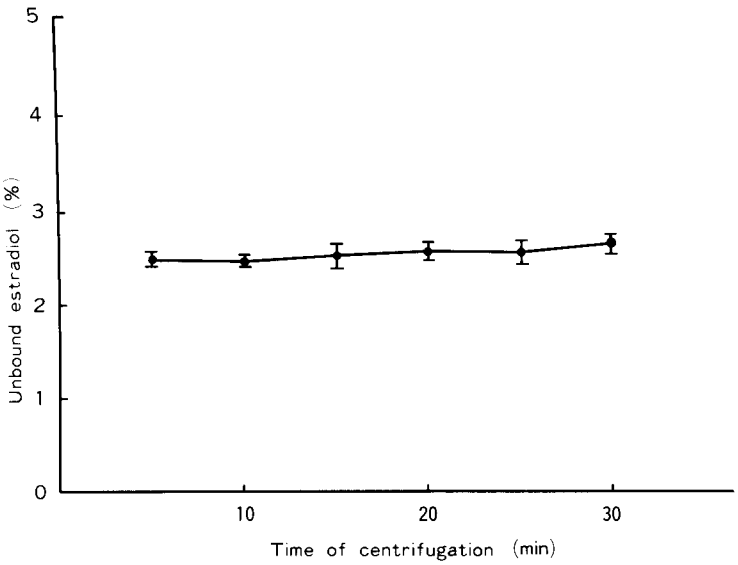

Fig. 1 Influence of various lengths of centrifugation time on the fraction of unbound estradiol. Each value is the mean of eight determinations. Standard deviations are represented by the bars.

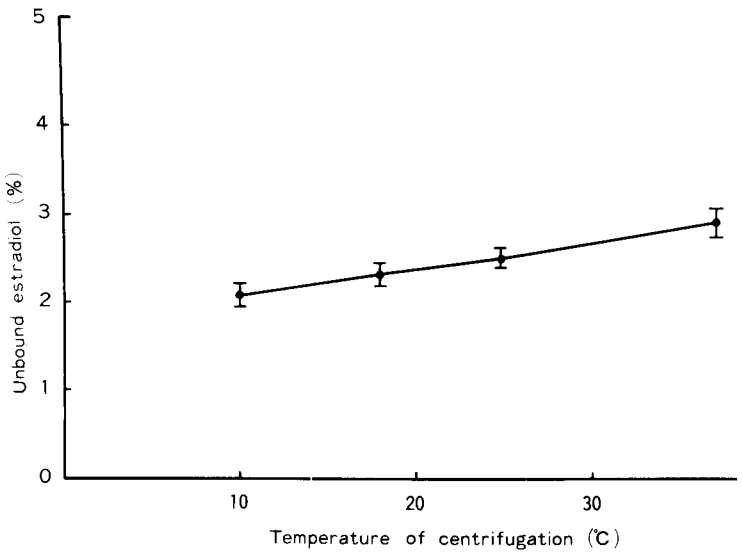

Fig. 2 Influence of temperature on the fraction of unbound estradiol, Other conditions are the same as those in the standard procedure. Each value is the mean of eight determinations, standard deviations are represented by the bars.

tures. A lower temperature for the procedure resulted in a lower percentage of unbound estradiol.

The percentage of unbound estradiol also varied with the pH of the sample (Fig. 3). Elevation of the pH brought about a higher fraction of unbound estradiol.

The determined percentage of unbound estradiol changed with even a slight degree of dilution (Table 1). When the dilution was larger than 1 : 1.10, correction of the percentage with the dilution factor alone gave lower than expected values.

The comparison of the radioactivity of the ultrafiltrate of the pre-dialyzed serum against that of the dialysate gave a ratio of 0.98

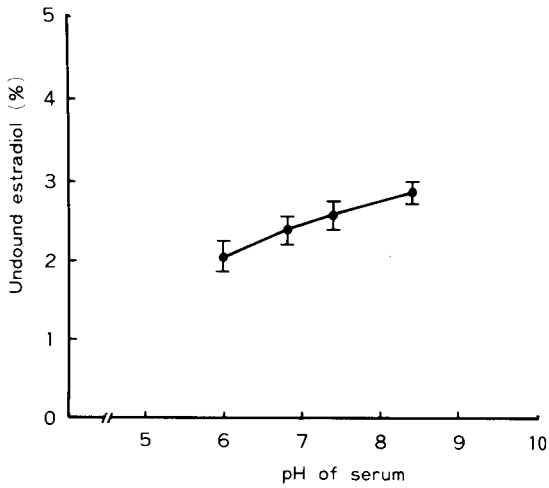

Fig. 3 Influence of pH on the fraction of unbound estradiol. Other conditions are the same as those in the standard procedure. Each value is the mean of eight determinations. Standard deviations are represented by the bars.

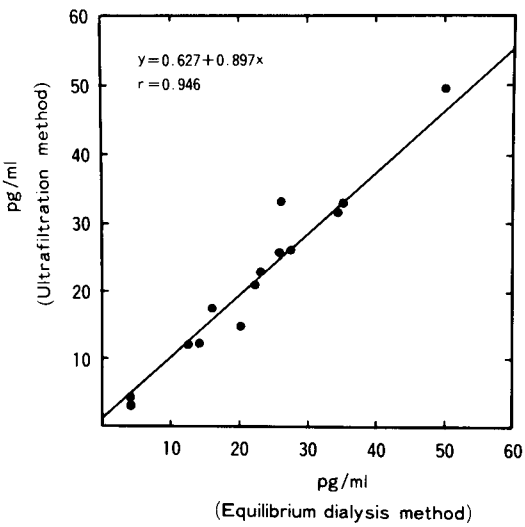

Fig. 4 Comparison of the unbound estradiol concentration determined by the equilibrium dialysis and ultrafiltration methods.

± 0.08 (mean $\pm$ SD) for 8 samples. Fig. 4 shows the result of a direct comparison between equilibrium dialysis and ultrafiltration. The unbound estradiol concentration determined and calculated by equation (4) showed a good correlation for 14 samples between the two methods ($r = 0.946$, $p < 0.01$).

Table 1 Effect of dilution upon the fraction of unbound estradiol

Dilution	Determined[a]	Corrected[b]
1.05	2.61 (0.06)	2.49 (0.06)
1.10	2.78 (0.12)	2.53 (0.10)
1.50	3.57 (0.09)	2.38 (0.06)
5	10.45 (0.49)	2.10 (0.10)
10	19.54 (0.88)	1.95 (0.08)
20	35.57 (1.47)	1.78 (0.07)

a: Percent of unbound estradiol in pooled serum was determined by the ultrafiltration method. Values, which are expressed as % of the total, are means of eight determinations. Standard deviations are shown in the parentheses.

b: Corrected by dividing the determined value by the dilution factor using equation (2).

The intraassay coefficient of this method was 7.5% for 10 measurements with pooled serum. The interassay coefficient was 6.2% for the same pooled serum measured 25 days apart.

The patients under study were grouped according to their menstrual date as shown in Table 2. The patients were gathered around the ovulation time because they were scheduled for observation of follicular growth or the presence of a corpus luteum. Patients in the follicular phase were divided into day 7-11 and day 12-14 groups, and patients in the luteal phase were divided into day 15-18 and day 19-28 groups.

Variations in the volume of peritoneal fluid and estradiol and progesterone concentrations in peritoneal fluid among the groups are shown in Table 2. An abrupt elevation in the peritoneal fluid progesterone and estradiol shortly after ovulation (day 15-18) was noticed. Reductions in the peritoneal fluid volume, estradiol and progesterone after day 19 were noticed, but the peritoneal fluid estradiol and progesterone concentrations were higher than those of the serum.

The percentage of unbound estradiol in serum did not significantly differ among the groups (Table 2). However, variation was noticed in peritoneal fluid. Before ovulation,

Table 2 Changes in estradiol and progesterone concentrations[a] in serum and peritoneal fluid

Details	Group				
	1	2	3	4	5
Day of cycle	7-11	12-14	15-18	19-28	LUF[b]
No. of patients	3	8	9	5	1
Serum					
Progesterone (ng/ml)	2.0 (1.6)	2.2 (1.4)	8.3 (5.3)	9.7 (2.9)	19.6
Estradiol (pg/ml)	103.7 (94.0)	248.1 (93.7)	126.8 (63.1)	93.6 (11.1)	89.0
Unbound estradiol (%)	2.5 (0.3)	2.6 (0.2)	2.6 (0.2)	2.6 (0.4)	2.2
Peritoneal fluid					
Volume (ml)	8.3 (3.2)	14.6 (5.5)	26.9 (16.0)	14.0 (8.6)	5.0
Progesterone (ng/ml)	2.7 (1.0)	2.9 (0.8)	148.2 (106.9)	18.5 (12.7)	25.1
Estradiol (pg/ml)	50.7 (67.0)	217.3 (98.7)	1331.6 (797.3)	108.8 (43.5)	86.0
Unbound estradiol (%)	4.3 (0.3)	4.5 (0.2)	3.8 (0.4)	3.5 (0.1)	4.5

a: Estradiol and progesterone concentrations were determined by radioimmunoassay. Values are means of values of patients in each group. Standard deviations are shown in parentheses.

b: A patient with luteinized unruptrued follicle (LUF) syndrome at day 17 of the cycle.

the percentage of unbound estradiol in peritoneal fluid averaged 4.3% and 4.5% for the day 7-11 and day 12-14 groups, respectively. On the other hand, after ovulation, 3.8 and 3.5% were calculated for the day 15-18 and day 19-28 groups. There was a significant difference ($p < 0.05$) in the fraction of unbound estradiol in peritoneal fluid between the day 12-14 and day 15-18 groups and between the day 12-14 and day 19-28 groups.

A patient diagnosed as having luteinized unruptured follicle (LUF) syndrome at day 17 was noticed to have a high level of serum progesterone but relatively low peritoneal fluid estradiol and progesterone level in peritoneal fluid in contrast with those of day 15-18 group patients. The fraction of unbound estradiol (4.5%) in peritoneal fluid was comparable with that of the follicular phase patients.

Discussion

The ultrafilter (Centrifree®) used in this study is disposable and thus eliminates the necessity of washing the membrane or the cells mentioned in some reports (13, 18). The filter did not adsorb estradiol, and good recovery of estradiol was obtained with this filter in a preliminary study.

When the fraction of unbound steroid is determined by equilibrium dialysis, the influence of the tracer impurity is usually not obvious. However, with ultrafiltration, the impurity of the tracer affects the fraction of unbound hormones to a great extent (13, 18). The authors purified the tracer estradiol by LH-20 chromatography immediately prior to use to eliminate the effect of the tracer impurity upon determining the fraction of unbound estradiol. Drying up of the tracer before the addition of the sample protects the sample from the influence of the solvents of the tracer and reduces the dilution.

Changing the volumes centrifuged did not have any effect upon the fraction of unbound estradiol determined. The volume of the ultrafiltrate obtained was usually 150-200 μl when a 0.5-ml sample was used. Therefore for simplicity of procedure, 0.5 ml was usually used the sample volume.

The temperature and pH of the sample during centrifugation are important in varying the unbound fraction of estradiol. Centrifugation under conditions similar to the

in vivo state, *i.e.*, at 37℃ and at pH 7.4, are thus mandatory.

Dilution of the sample affects the determined fraction of unbound hormones to a great extent. Even a small dilution can cause a pronounced change in the percentage of unbound estradiol, and correction by dividing by the dilution factor alone does not give consistent results for varying degrees of dilution. As the dilution increases, the corrected percentage of unbound estradiol decreases if the correction is calculated by simply dividing the calculated percentage by the dilution factor only. For a dilution of more than 1 : 1.10, simple correction with the dilution factor only carries the risk of underestimating the actual percentage of unbound estradiol. This type of underestimation is also seen when determining the unbound fraction of other protein-bound hormones (13, 20-22). Therefore, it is indispensable to carry out the procedure with as little dilution as possible.

The pH of the serum usually rises to over 8.2 after separation and storage due to the escape of CO_2. The addition of highly concentrated HEPES buffer following the standard procedure kept the pH of the sample at 7.4 with only a 1.05-fold dilution and minimized the effect of dilution on the fraction of unbound estradiol.

A good correlation between the present ultrafiltration method and equlibrium dialysis was recognized and proved the validity of this procedure. Besides, this method is quite simple and rapid in contrast with equilibrium dialysis.

The volume of peritoneal fluid and serum and peritoneal fluid estradiol and progesterone levels of the patients studied in various phases are similar to values observed in other series reported in the literature (2, 5, 6, 21, 22). The percentage of serum estradiol was similar to the percentage reported in the literature (25) and showed no significant variation during the menstrual cycle. On the other hand, variation of the fraction of unbound estradiol in peritoneal fluid among various groups was disclosed although no significant difference was noticed in the serum at various phases.

Peritoneal fluid originates mainly from the ovaries throughout the menstrual cycle (26). Some form of material exchange is known to be present between the peritoneal fluid and the intravascular compartments (27). When the growing follicle extends to the ovarian surface, follicular fluid is separated from the pelvic cavity by the thin follicular wall composed of the membrana granulosa, tunica albuginea and a layer of cuboidal cells. This exposed area of the follicular wall also allows the passage of some materials into the pelvic cavity. Subsequent to the rupture of the follicle following ovulation, this follicular barrier disappears and the resultant corpus rubrum and corpus luteum come into direct contact with the pelvic cavity.

The significant difference between the fraction of unbound estradiol of the patients before ovulation (mean of 4.5% of the day 12-14 group) and that after ovulation (mean of 3.8% of the day 15-18 group and 3.5% of the day 19-28 group) is postulated to be due to a different mechanism of ovarian steroid secretion into the pelvic cavity.

Sex steroids inside the follicle are usually bound to a large molecular protein (such as CBG, TeBG, albumin, *etc.*) (2) which can not pass through the follicular wall. Unbound estradiol enters the peritoneal cavity mainly by transudation, and results in a higher percentage of unbound estradiol and a lower percentage of protein-bound estradiol in peritoneal fluid.

In the luteal phase, the follicle turns into corpus rubrum and later into corpus luteum, with a rich blood supply on the surface which allows active exudation from the surface to take place readily. Simultaneous active secretion of the protein and steroids from the

corporeal surface predominates. Such secretion was verified by the occasional observation during laparoscopic examination of dripping from the ovarian surface shortly after ovulation and by the abrupt increase in peritoneal fluid volume together with the increase in the progesterone content of the day 15-18 group.

Before ovulation, the level of estradiol in peritoneal fluid was lower than that in the serum. Although over 1.5 times the fraction of unbound estradiol was noticed in proliferative phases, the unbound estradiol concentration in peritoneal fluid was still quite low. After ovulation, the percentage of unbound estradiol in peritoneal fluid was reduced, but the content of unbound estradiol increased, especially around the stage of fertilization to implantation. The physiological significance of this phenomenon, however, is unclear and awaits further investigation.

In the patient with LUF syndrome, the fraction of peritoneal fluid unbound estradiol was similar to that of the patients in the follicular phase in spite of the presence of luteinization of the follicle. This similarity is assumed to result from transudation of the follicular steroids into the pelvic cavity instead of exudation seen in normally ovulated patients in the luteal phase. The result is in agreement with this postulation, and the peritoneal fluid unbound estradiol fraction is expected to serve as a parameter for biochemically diagnosing LUF syndrome.

In conclusion, ultracentrifugation with the Centrifree® filter proved to be valid for determining the fraction of unbound estradiol in body fluids under conditions similar to those *in vivo*. The method is simple and rapid. The application of this method to infertility patients did not demonstrate a variation in serum estradiol throughout the menstrual cycle. However, a significant difference was noticed to exist in the peritoneal fluid estradiol between the follicular and luteal phases of the menstrual cycle. This difference is thought to be due to the difference in the mechanism of secretion of sex steroids of ovarian origin. Lack of exudation from the ovarian surface due to the persistence of the follicular wall in the patient with LUF syndrome might explain the higher percentage of unbound estradiol in peritoneal fluid.

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