Radioimmunoassay for Luteinizing Hormone in Human Plasma or Serum: Physiological Studies *

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Summary. It is not practical to quantitate gonadotropin in the blood of normal men and women by utilizing bioassays. We have developed a method for sensitive, precise, and specific radioimmunoassay of luteinizing hormone (LH) in human serum or plasma. Antisera were developed against human chorionic gonadotropin, and one of these was selected for extensive cross-reaction with human LH. Highly purified LH was radioiodinated by the method of Greenwood, Hunter, and Glover. Separation of antibody-bound from free LH-^{131}I was accomplished by a double antibody technique. Dose-response curves for the purified LH, an impure urinary LH preparation, pituitary powder, and LH in plasma were all identical. Immunoassay and bioassay of impure urinary and pituitary gonadotropin preparations in terms of a common standard resulted in an index of discrimination of close to unity. LH levels in plasma from 32 adult men and 30 women outside the midcycle ranged from 0.6 to 3.2 mμg per ml (1 mμg of our laboratory LH standard is equivalent to 8 mU of the Second International Reference Preparation of Human Menopausal Gonadotropin). Levels were remarkably constant in men from day to day and in women except at midcycle, when a sharp peak occurred lasting less than 24 hours. In all women studied who had a midcycle LH peak, mean plasma LH levels during the follicular phase of the menstrual cycle were higher than mean values obtained during the luteal phase. Prepubertal children had detectable plasma LH, and mean values were only slightly less than in adults. Plasma from castrate men or women or postmenopausal women contained 4.5 to 10.5 mμg per ml. Clomiphene treatment of four men resulted in a doubling of plasma LH in 5 days.

Introduction

Wide, Roos, and Gemzell (1), using an immunoassay based on hemagglutination inhibition, first reported that human luteinizing hormone (HLH) cross-reacts with human chorionic gonadotropin (HCG) for binding to antibodies directed against HCG. In 1964 Paul and Odell (2) first developed a radioimmunoassay for HCG. Later we (3) developed a radioimmunoassay for

HLH by taking advantage of the cross-reaction between HLH and HCG and the availability of a highly purified preparation of HLH suitable for radioiodination.

In 1965 Midgley and Ram (4) published an abstract of methods of radioimmunoassay for HLH, and subsequently Midgley (5) and Franchimont (6) published reports of methods for radioimmunoassay for HLH. We wish to report details of our methodology and some results of physiological studies.

Methods

Preparation of antisera to HLH (anti-HLH). All immunizations were performed by dissolving the antigen

in saline and emulsifying this solution with an equal volume of complete Freund's adjuvant.[1] Three ml of this mixture was given subcutaneously in two sites to each white New Zealand rabbit. Three such injections were administered 10 days apart. Animals were bled periodically by cardiac puncture starting 10 days after the last injection. Two gonadotropin preparations were used to prepare the antisera: *1)* a preparation from urine of pregnant women[2] (3,000 IU was administered in each injection; antisera from 12 animals were studied), and *2)* a preparation from urine of castrate men[3] (12.5 mg was administered at each injection, and antisera from six animals were studied). All antisera were screened for titer and ability to bind a tracer amount of human luteinizing hormone labeled with [131]I (LH-^{131}I) by incubating tenfold dilutions of the antisera with 0.05 mμg of LH-[131]I. Antisera that bound more than 70% of this antigen at greater than 1:10,000 final dilution were further screened by running abbreviated dose-response curves of unlabeled HLH in the radioimmunoassay system. An antiserum directed against HCG was finally selected to develop the assay described.

Sheep antirabbit antisera were prepared by dissolving 100 mg of rabbit γ-globulin[4] in 5 ml of 0.9% NaCl and mixing with 5 ml of complete Freund's adjuvant. Ten ml of this mixture was injected subcutaneously into a sheep on the same schedule as described for rabbits. Two sheep were immunized and periodic bleeding was performed starting 10 days after the last injection. Every batch of antiserum was titered for ability to precipitate the antibody-bound HLH-^{131}I mixture described later. An "excess" (usually 150 μl) was used as the second antibody for assay purposes.

Radioiodinated human luteinizing hormone. One mg of highly purified HLH was utilized in the development of this assay. This particular batch of HLH had not been bioassayed because insufficient material was available, but with similar methodology HLH preparations containing 6,400 IU per mg[5] had been previously prepared (7). The HLH was dissolved in phosphate saline buffer (0.01 M phosphate, 0.15 M NaCl, pH 7.8) in concentrations of 0.1 mg per ml and frozen in aliquots of 0.2 ml for each assay. One or 2 μg of this material was radioiodinated to specific activities of 200 to 500 μc per μg by the method of Greenwood, Hunter, and Glover (8). LH-^{131}I was separated from inorganic ^{131}I by passing through a G75 Sephadex column (1.0 × 15 cm). The trailing edge of the earliest peak was used in the assay. LH-^{131}I was always utilized within 24 to 48 hours of preparation.

Performance of assay. The following buffer was used for all dilutions: 0.01 M phosphate, 0.15 M NaCl, 0.1%

sodium azide, 2.5% normal rabbit serum, pH 7.8. All reagents were added to 1.0- × 7.5-cm tubes in the following order: *1)* buffer to make a total volume of 1.0 ml, *2)* 100 μl of 0.1 M EDTA, pH 7.6, *3)* 1.0 to 300 μl of plasma to be assayed (or of "standard" hormone), *4)* 100 μl containing 0.05 to 0.15 mμg of HLH-^{131}I, and *5)* 100 μl of antisera suitably diluted, usually 1:30,000 (final dilution, 1:300,000).

Complete standard dose-response curves were run in all assays, and for this purpose known amounts of HLH were added instead of the plasma. All tubes were incubated for 5 days at 4° C, at which time 150 μl of the second antibody was added to each tube and the mixture incubated 24 hours longer at 4° C to achieve separation of antibody-bound from free HLH-^{131}I, according to the method of Feinberg (9) as modified by Morgan, Sorenson, and Lazarow (10). Tubes were then centrifuged at 500 g and the supernatant was removed by suction. Radioactivity was quantitated in an automatic gamma spectrometer,[6] and all results were expressed as per cent of counts precipitated. Zero per cent was defined as no HLH-^{131}I bound to antibody. Two to 5% of HLH-^{131}I was trapped in the precipitate nonspecifically, the value of these counts being obtained by quantitating precipitated counts in tubes containing no antibody. These counts could be removed by washing the precipitate, but this did not contribute significantly to precision of the assay, and this step was therefore omitted. The counter was then set so that these counts were automatically subtracted (as background). One hundred per cent was defined as the number of counts precipitated in tubes containing HLH-^{131}I and antibody, but no unknown or standard LH. All tubes were counted for the time required for the "100% tubes" to register 10,000 counts. Thus any tubes with unlabeled HLH present had HLH-[131]I "displaced" from antibody, and the result was less than 10,000 counts precipitated. In this manner all results were expressed in relation to 10,000 counts and by shifting the decimal two places to the left were expressed as per cent. This procedure greatly simplified data processing.

Bioassay. Several samples were simultaneously bioassayed and immunoassayed in terms of the same human standard. Bioassays for HLH were done by means of the ventral prostate weight in hypophysectomized immature male rats (11). It is important to note that a human LH standard is required for the purposes of radioimmunoassay because LH of other species reacts poorly or not at all in the assay. Pergonal 1958[7] was adopted as our laboratory standard for both bioassays and immunoassays. After repeated potency estimates in the radioimmunoassay 1 μg of this laboratory standard was defined as equivalent to 2 mμg of purified HLH and as equivalent to 0.016 IU[8] of LH.

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[1] Difco Laboratories, Detroit, Mich.

[2] APL, Ayerst, New York, N. Y.

[3] HPGUE, National Institutes of Health study section.

[4] Hyland Laboratories, Los Angeles, Calif.

[5] In terms of the Second International Reference Preparation of Human Menopausal Gonadotropin (2nd IRP HMG).

[6] Picker X-Ray Corp., White Plains, N. Y.

[7] Purchased from Instituto Farmacologico, Serono, Rome, Italy.

[8] In terms of international units of the 2nd IRP HMG.

Results

Ninety-five per cent or more of the HLH-^{131}I was precipitable in the presence of excess anti-HLH. Figure 1 demonstrates a semilogarithmic dose-response curve for the purified HLH in the radioimmunoassay. The minimal amount detectable was 0.15 mμg per ml (1.2 mU) of plasma when 300 μl was assayed in each milliliter of reaction mixture. Also shown in Figure 1 on the same semilogarithmic scale is a dose-response curve for our laboratory standard (Pergonal). Note that this relatively impure gonadotropin preparation containing both follicle-stimulating hormone (HFSH) and HLH gives the same dose-response curve as the purified HLH with the exception that 504 times as much is required to give the same response. In addition, a dose-response curve for acetone-dried human pituitary powder is shown. This material also gave a dose-response curve similar in shape to that of the purified HLH, but 1,000 times as much material was required for the same response. Figure 2 depicts dose-response curves for highly purified HLH and for plasma from a postmenopausal woman. Note the identical shapes of these dose-response curves.

Partially purified and highly purified prepara-

FIG. 2. SEMILOGARITHMIC PLOT OF DOSE-RESPONSE CURVES FOR HIGHLY PURIFIED HLH AND FOR THE HLH CONTENT OF PLASMA FROM POSTMENOPAUSAL WOMEN. The two points at 20 μl are duplicate determinations.

tions from a number of sources have been assayed by this technique to assist in evaluation of specificity. Highly purified HFSH, which initially assayed biologically at 100 IU per mg,[9] gave a dose-response curve identical to that of the HLH except that 100 times as much material (by weight) was required to give the same response, a phenomenon probably explained by a 1% contamination of the HFSH with HLH. Human thyrotropin (HTSH), which initially assayed at 20 U per mg, also gave a dose-response curve identical to the HLH, but 20 times as much was required for the same response, a phenomenon probably explained by 5% contamination of the HTSH with HLH. The values for per cent contamination are similar to those obtained for both HTSH (12) and HFSH (13) by bioassay. The dose-response curve for human chorionic gonadotropin is also identical to HLH. As little as 0.002 IU[10] may be detected. This is 50 times more sensitive than the mouse uterine weight assay (14). It is interesting that monkey chorionic gonadotropin shows minimal cross-reaction in this assay for HLH, as much as 100 IU giving the same response as 0.01 IU of HCG. The differences in monkey and human chorionic gonadotropin will be presented in detail in a separate publication.
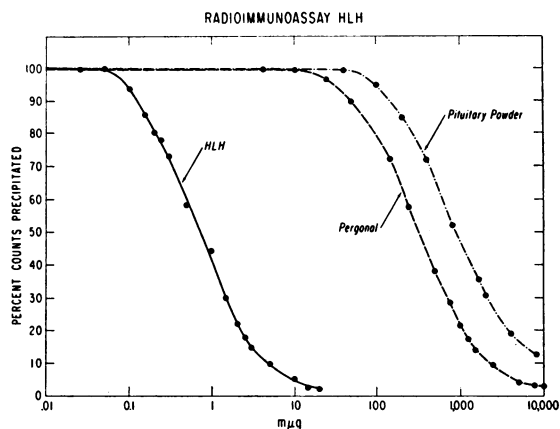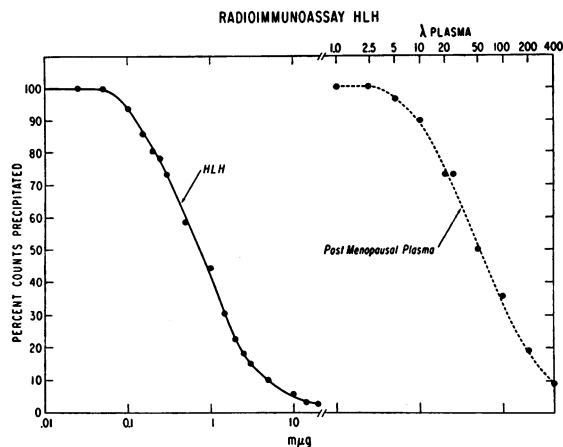

FIG. 1. SEMILOGARITHMIC PLOT OF DOSE-RESPONSE CURVES FOR HIGHLY PURIFIED HUMAN LUTEINIZING HORMONE (HLH), AN IMPURE URINARY PREPARATION (PERGONAL) CONTAINING BOTH FOLLICLE-STIMULATING HORMONE AND HLH, AND ACETONE-DRIED HUMAN PITUITARY POWDER. Five hundred times as much Pergonal and 1,000 times as much pituitary powder were required to give the same response. One mμg is equivalent to 8 mU of the Second International Reference Preparation of Human Menopausal Gonadotropin.

[10] The international unit of HCG is different from the international unit of the 2nd IRP HMG.

TABLE I

Comparison of immunological and biological activity of human luteinizing hormone

Material	Biological activity*		Immunological activity	
Argenio ⎱ Burus ⎬ pool Cortez ⎰	683	μg/24 hours	570	μg/24 hours
Dean pool	450	μg/24 hours†	207	μg/24 hours
Kempanien pool	1,125	μg/24 hours†	720	μg/24 hours
Denegal pool	412	μg/24 hours†	660	μg/24 hours
Moreland powder	0.75	μg/mg†	0.445	μg/mg
Pituitary powder	0.23	mg/mg	0.30	mg/mg
HPGUE‡	0.25	mg/mg	0.1	mg/mg
Postmenopausal plasma	2.4	mg/L (McArthur)	3.7	mg/L
	5.4	mg/L (Apostolakis)	(2.3–5.1)	

* In terms of Pergonal 1958.
† One on 3 assay design.
‡ National Institutes of Health study section.

Several impure gonadotropin preparations (containing both LH and FSH) were simultaneously assayed in the radioimmunoassay and in the prostate weight assay in terms of a common standard. Results are presented in Table I. For several samples only enough LH was present to permit a 1 on 2 bioassay design. The index of discrimination was close to unity in all cases, and in all cases the 95% confidence limits of the potency estimate by bioassay included the potency estimate by immunoassay.

Plasma from a number of sources has been examined for HLH content, and a scattergram of some of these data is presented in Figure 3. Plasma from eugonadal men and women except at midcycle contained an average of 1.8 mμg per ml (range from 0.8 to 3.2). In men plasma values for HLH appeared fairly constant. Samples from four men under normal activity were obtained every 6 hours for 4 days and for two of these same men at times when 24-hour bed rest was maintained. No diurnal variation of HLH has been observed under any of these conditions. Plasma from children of both sexes between the ages of 8 and 12 years contained an average of 1.6 mμg per ml (0.6 to 2), and plasma from children 1 to 8 years of age contained an average of 1.0 mμg per ml (0.6 to 1.6). The differences among all four of these groups are not statistically significant. The presence of gonadotropin in plasma from prepubertal children is discussed in more detail later.

Plasma from postmenopausal women and castrated men or women contained from 6 to 12 mμg per ml. There was no overlap in LH values from eugonadal men and women outside the midcycle. A single LH determination serves to differentiate primary from secondary gonadal failure in all cases we have yet seen.

Figure 4 depicts the plasma LH levels determined daily in a eugonadal woman throughout the menstrual cycle. Note that blood levels were
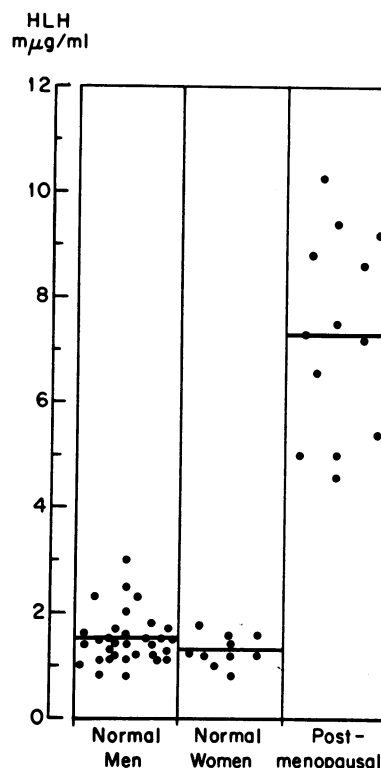

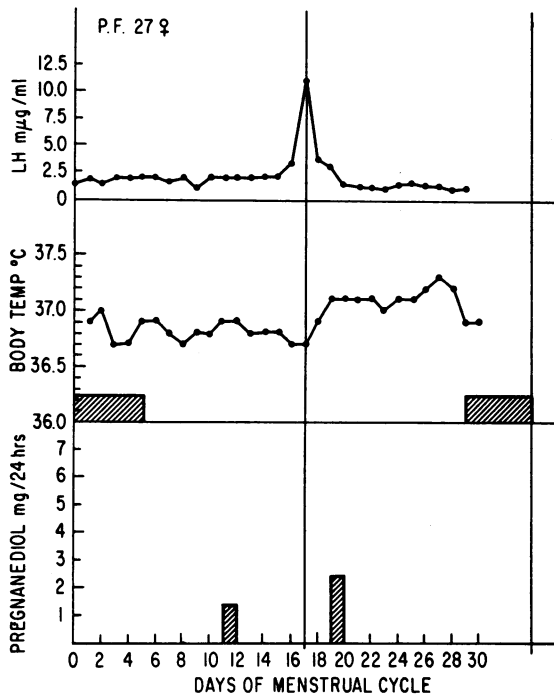FIG. 3. SCATTERGRAM OF HLH VALUES IN PLASMA FROM EUGONADAL MEN AND WOMEN AND FROM POSTMENOPAUSAL WOMEN.

FIG. 4. PLASMA LH LEVELS MEASURED DAILY THROUGH-
OUT A MENSTRUAL CYCLE IN A 27-YEAR-OLD WOMAN. Men-
strual flow is shown by the shaded bars under the body
temperature curve. Note the relative constancy of LH
levels except at midcycle. Note also that the mean LH
levels during the follicular phase are higher than during
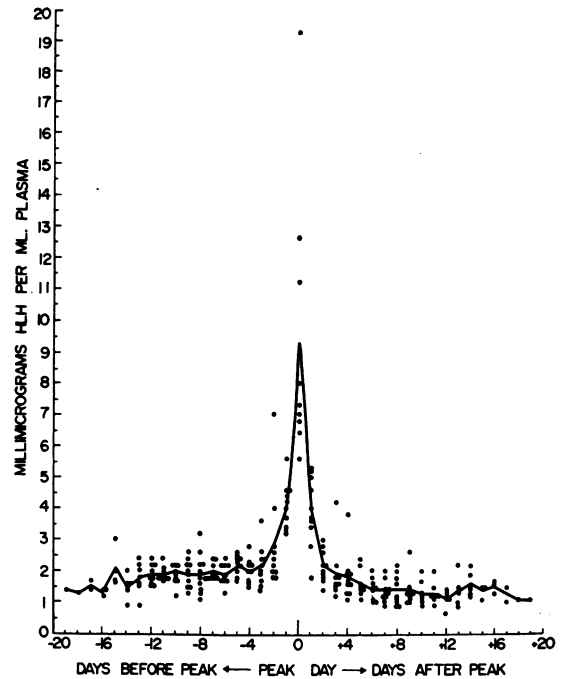the luteal phase.

FIG. 5. SCATTERGRAM OF PLASMA HLH VALUES MEA-
SURED DAILY IN TEN EUGONADAL WOMEN THROUGHOUT
THE MENSTRUAL CYCLE. Sample collection was initiated
between days 1 and 3 of one menstrual period and termi-
nated on the last day of the following menstrual period.
In any one patient, HLH values during the follicular
phase always averaged higher than levels during the
luteal phase. Data from each patient were plotted by
centering all midcycle peaks.

fairly constant except at midcycle, where a sharp
peak occurred. The mean plasma level of LH was
higher during the follicular phase than during the
luteal phase. Also shown are basal body tem-
peratures and pregnanediol excretion. We have
followed such changes through a menstrual cycle
in 30 women, and the midcycle peak was typically
seen at the suspected time of ovulation in 27. In
three women anovulatory cycles were suspected
because of a delay of 10 to 20 days in arrival of
expected menses, and no midcycle peak was ob-
served. Figure 5 depicts a scattergram of data
obtained by measuring plasma LH daily in ten
women throughout the menstrual cycle. A sharp
LH peak ranging from 5.1 to 19.2 mμg per ml
(71.4 to 268.8 mU per ml) and occurring as early
as day 13 and as late as day 21 was seen during
each cycle. Mean plasma LH levels during the
follicular phase averaged 2.0 ± 0.03 (SE),
whereas during the luteal phase they averaged
1.5 ± 0.08 mμg per ml. In all patients examined

the LH values obtained during the luteal phase
were less than those obtained during the follicular
phase. Treatment of 11 of these women with an
oral contraceptive agent (Enovid E, 2.5 mg per
day) for the next consecutive cycle after the one
reported resulted in a complete suppression of this
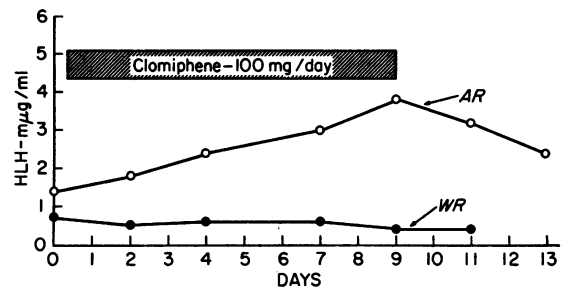LH peak.

FIG. 6. RESPONSE TO CLOMIPHENE TREATMENT OF TWO
BROTHERS. W. R. had hypogonadotropic hypogonadism;
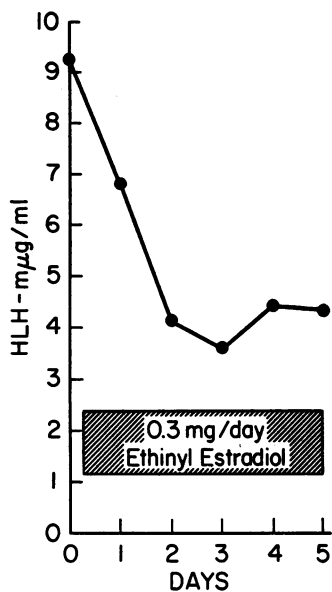A. R. was eugonadal.

FIG. 7. PLASMA LH LEVELS IN A POSTMENOPAUSAL
WOMAN TREATED WITH ESTROGEN. Note that LH levels
fell by 50% or more but plateaued at values about twice
those seen in eugonadal men and women except at mid-
cycle.

Four normal men have been treated with clomi-
phene in an attempt to raise plasma LH levels.
In all four normal men plasma LH rose two to
four times after 5 days' treatment. Figure 6 de-
picts data from two brothers; one suffered from
hypogonadotropic hypogonadism, a diagnosis
based on physical examination, testicular biopsy,
bioassay of ten serial 24-hour urine collections,
and ability to increase plasma testosterone levels
in response to treatment with human chorionic
gonadotropin. His plasma LH level was 0.8 mμg
per ml (a low normal value). Treatment for 5
days with 100 mg per day of clomiphene failed
to change plasma LH values. In contrast, his
eugonadal brother simultaneously treated with the
same medication had a progressive elevation in
plasma LH during the treatment period. Further
study of these effects of clomiphene on plasma LH
is in progress.

Figure 7 depicts data obtained by treatment of
a 58-year-old postmenopausal woman with 0.3 mg
of ethinyl estradiol per day for 5 days. Note that
initial LH levels were 9.2 mμg per ml and that they
fell under treatment to levels of 4 mμg per ml
(still above levels in eugonadal men or women out-
side the midcycle) and leveled off at this point.

Discussion

With concentrations of large volumes of urine
or plasma and bioassays, a great deal of informa-
tion has been gained concerning levels of HLH in
human subjects. For example, McArthur and
her associates (15) and Apostolakis (16) ex-
tracted up to 1,000 ml of blood from postmeno-
pausal women and quantitated HLH by bioassay.
More recently Louchart, Truffert, and DeCourt
(17) and Keller (18) measured HLH in plasma
of normal men. The potency estimates reported
by the latter two groups differed by a factor of
40 to 50. Louchart and his colleagues (17) also
reported results of bioassay for HLH in plasma
from three normal women in early, middle, and
late periods of menstrual cycles. Midcycle val-
ues were higher than values obtained at other
times during the cycle. Using concentrates of
urine, McArthur, Worcester, and Ingersol (19)
quantitated HLH throughout the menstrual cycle
in normal women. These workers demonstrated
a midcycle peak of HLH excretion. Subse-
quently, these findings were confirmed by Fuku-
shima, Stevens, Gantt, and Vorys (20), Becker
and Albert (21), and Rosemberg and Keller (22).

Utilizing immunoassays based on hemagglutina-
tion-inhibition and concentrates of urine, several
workers have measured HLH during the men-
strual cycle. Wide and Gemzell (23), Butt, Cun-
ningham, and Hartree (24), and Sato, Greenblatt,
and Mahesh (25) demonstrated midcycle increases
in the excretion of immunoreactive material. Our
results with radioimmunoassay confirm that the
increase in LH excretion at midcycle is associated
with a corresponding peak of HLH in plasma.
This peak of LH has a value outside the total
range of values obtained during other portions of
the menstrual cycle. For the first time, it has
been shown that plasma levels obtained during
the luteal phase are lower than those in the follicu-
lar phase. Similarly, confirmation of the elevated
levels of HLH found by bioassay of plasma from
postmenopausal women has been obtained. Fur-
thermore, assay of eight preparations containing
HLH by biological and radioimmunological means
in terms of a common standard has revealed a
close correspondence of values. These observa-
tions coupled with the similarity of our results
to those of Midgley (5) strongly suggest that the
radioimmunoassay for HLH is a reliable and spe-

cific method for quantifying this hormone in plasma. This assay has the obvious advantage of requiring only small volumes of plasma and makes possible repetitive determinations at short time intervals in a single individual. It should come as no surprise that immunoreactive HLH was detectable in plasma from prepubertal children, since LH activity has been previously demonstrated by bioassay of concentrates of urine from prepubertal children (26).

The presence of gonadotropins in urine and plasma prepubertally suggests that a dynamic pituitary-gonadal interreaction may exist before puberty in man. Such a relationship has been demonstrated in animals. Plasma from prepubertal rats contains LH that is detectable by its effects on a parabiont hypophysectomized partner (27). When immature rats are joined in parabiosis and one partner is castrated, the other undergoes precocious sexual development (28). This sexual development may be prevented by treatment of the castrate animal with estrogenic steroids in doses that are too small to have any effect on the sex accessories (29). A number of studies are in progress in our laboratory to further elucidate mechanisms of puberty. We have recently observed two prepubertal girls, one age 11, one age 12, with castrate levels of plasma LH; both girls have been found to have no gonadal tissue.

The facts that LH is detectable in blood of prepubertal children and that the levels are only slightly lower than in adults challenge our present concepts of mechanisms of puberty. It appears that puberty is not simply caused by onset of gonadotropin secretion, which in turn stimulates gonadal steroid secretion, but by a more complex phenomenon that involves a dynamic pituitary-gonadal interrelation before sexual maturation.

The preliminary observations on elevations of plasma LH in response to clomiphene treatment indicated that this may be usable as a test of pituitary LH "reserve" in a fashion similar to the insulin hypoglycemia (20) or pyrogen-induced (21) elevation of growth hormone or to the metopirone release of ACTH (22).

Acknowledgments

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