New Field Techniques for Detection of Female Reproductive Status

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I. INTRODUCTION

Field studies in reproductive ecology and demography are frequently hampered by difficulties in accurately determining prevalence of pregnancy and age at menopause, although both are important for characterizing fertility parameters. Recent semiquantitative techniques for the measurement of gonadotrophins in urine have allowed for accurate determination of reproductive status using non invasive, non disruptive methods. Our laboratory has identified two low technology methods for the detection of human chorionic gonadotrophin (hCG) and lutenizing hormone (LH) in urine that provide reliable and accurate assessment of pregnancy and menopause under the most stringent of field conditions.

Pregnancy is ascertained using an unusually sensitive and specific qualitative method that measures hCG in urine with a double monoclonal antibody enzyme-linked technique. Reliability of detection at 20 mIU/mL is 100%, while that at 10 mIU/mL is 60%. Detection of menopause is achieved using a technique designed to identify the mid-cycle LH peak. It allows a semiquantitative determination of urinary LH and involves a double monoclonal antibody enzyme-linked method specific for intact LH. The test is sensitive to 20 mIU/mL. If LH is elevated, the measure is repeated 5-7 days later. Menopausal status is inferred from a second elevated LH level. These methods may be particularly helpful in demographic studies investigating reproductive status in women at various stages of the reproductive life cycle.

II. SAMPLE COLLECTION

Both methods were tested in the laboratory and among the Hadza, a recently settled foraging society in Northern Tanzania that are the subject of ongoing anthropological investigation (Blurton Jones 1989, Hawkes 1989). Urine samples were collected from 190 Hadza women; of these, we have reliable ages (based on a ranking system) on 103 women. 116 women were tested for pregnancy, 82 were tested for LH.

add more info & cautions on reliability

A. Sampling Strategy

1. **Pregnancy Tests:** All women past menarche up to age 60 were tested for pregnancy with the Tandem Icon II hCG assay.

Icon II membrane and the ß- specific antibodies chemically linked to the enzyme alkaline phosphatase. The amount of enzyme immobilized on the test zone thus varies directly with the amount of hCG present. After removal of the unbound enzyme-linked antibodies, exposure of the bound complex to the enzyme substrate, indoxyl phosphate, results in blue color formation relative to the amount of substrate turnover. The test result is interpreted in comparison to the color development found at the positive reference control site. Specimens containing 50 mIU hCG/mL yield a color equivalent or darker than the reference; those specimens containing less than 50 mIU will appear lighter than the reference; while those without hCG will remain white.

B. **Reagents**

The kit (available from Hybritech) contains the following:

1) 48 testing cartridges with a specialized "ImmunoConcentrator" membrane containing immobilized mouse monoclonal antibodies
2) Antibody Conjugate containing mouse monoclonal IgG (anti-hCG) conjugated to bovine alkaline phosphatase
3) Substrate Reagent (indoxyl phosphate)
4) Wash Concentrate containing buffer and 0.3% sodium azide
5) 48 transfer pipettes.

Kits are stable at ambient temperatures of 15-30°C. Positive and negative urine controls are available separately from Hybritech.

C. **Assay Procedure**

The assay proceeds as follows:

1) Five drops (250 µL) of urine specimen are pipetted onto the Icon II cylinder membrane.
2) Three drops of antibody conjugate are added in rapid succession.
3) Reagents are left to incubate for 1 minute.
4) After the incubation period, the cylinder is rinsed with 500 µL wash solution.
5) Three drops of substrate reagent are then added to the Icon II cylinder membrane and allowed to incubate for two minutes.
6) After the final incubation, 500 µl of wash solution is again added to stop the reaction.

When hCG is present, the test cylinder will produce a circular blue spot at the center of the icon, below the reference spot.

D. Assay Sensitivity and Detection Limit

Reliability of detection of hCG with the Tandem Icon II at urine concentrations of 20 mIU/mL is 100%, while that at 10 mIU/mL is 70%. Clinical testing of the assay showed that the Tandem Icon hCG method agreed with the quantitative hCG IRMA in 99.6% of the urine samples tested (Strobel, Rinke, and Hussa 1985). The Icon II is highly specific for hCG; there is no interference of test results in the presence of homologous hormones (LH, TSH, FSH). Moreover, test results are not effected by the presence of many common drugs including acetaminophen, caffeine, salicylic acid, ampicillin, or tetracycline.

In pregnancy, hCG rises rapidly: it begins to be produced at 4-6 days post-conception, and increases exponentially in the early weeks of pregnancy (Figure 1). With the Icon II test, hCG should be detectable 7 - 10 days after conception.

IV. DETECTION OF MENOPAUSE

Assessment of reproductive lifespan is critical to any demographic study, yet surveys are frequently impeded by the difficulty in assessing menopausal status (Gray 1976). The actual median age of menopause in a population is difficult to determine because of early cessation of childbearing and the problem of ascertaining which menstruation is indeed the last one. Further, little is known about the factors that affect reproductive senesence in women, but nutrition and other environmental variables are suspected to play a role (Brand and Lehert 1978). Few studies have incorporated physiological measures in their cross-cultural investigations of menopause.

We have identified a preparation that assesses menopausal status through the semiquantitative measure of urinary LH. LH begins to rise about 12 months before menopause (Rannevik et al. 1986), and post menopausal LH levels reach a sustained peak that is significantly greater than the pre-menopausal level (Chakravarti et al. 1976). Two to three years after menopause, serum concentrations of LH are higher than during the follicular phase in premenopausal women; LH levels gradually decrease in women 20 to 30 years past the menopause (Chakravarti et al. 1976). (See Figures 2 and 3.)

Although the kit is designed to detect the mid-cycle LH surge, given that menopause entails chronic elevation of LH of a magnitude greater than the surge, it can also be used to detect menopause. The main potential for error arises from ambiguity over whether a woman is experiencing an ovulatory LH surge or is menopausal. This difficulty can be circumvented by retesting at a 5- to 7-day interval

after the first positive result. A second elevated reading indicates a menopausal condition, while a negative result indicates that the woman is cycling.

A. **The OvuKit LH assay (Monoclonal Antibodies)**

The OvuKit LH assay allows for visually interpreted, semiquantitative urinary LH detection that can be performed rapidly without special instrumentation. The assay detects urinary LH by using two monoclonal antibodies which react to two different regions of the LH molecule; antibodies are directed against the ß- and α -subunit, respectively. The α– subunit-specific antibody is immobilized on a white plastic dipstick while the β -subunit-specific antibody is linked to the enzyme conjugate. During the assay, the LH molecules are sandwiched between the dip stick-bound antibodies and the enzyme linked antibodies contained in the antibody conjugate. A color developer containing enzyme substrate, bromochloroindoxyl phosphate, becomes blue in the presence of the sandwiched enzyme remaining on the dip stick after a wash step. Blue color forms on the tip of the stick in proportion to the concentration of LH in the urine. The test result is interpreted in reference to a control stick assayed using water and a standard LH dose (35 ± 5 U/L) that is provided with the kit.

As Figures 4 and 5 demonstrate, the magnitude of LH involved in a mid-cycle surge and in menopausal transition are equivalent, in urine and in serum. Therefore, a measure designed to detect the former should also detect the latter condition.

B. **Reagents**

The kits (available from Monoclonal Antibodies) contain six or nine tests. Each test utilizes:

1) A dip stick with immobilized α -subunit-specific human glycoprotein hormone
2) One tube of lyphilized enzyme conjugate containing ß- specific antibodies linked to alkaline phosphatase
3) A small plastic vial containing conjugate buffer
4) One tube containing substrate solution (bromochloroindoxyl phosphate)

C. **Assay Procedure**

The assay proceeds as follows:

1) The contents of the vial containing 0.5 mL of conjugate buffer are emptied into a labelled tube containing dry enzyme conjugate.

2) The plastic vial, now empty, is used to pipette ten drops (approximately 0.5 mL) of urine into the labelled tube.
3) A corresponding test stick is then added, and used to mix the reagents for one minute.
4) Reagents are left to incubate for 30 minutes.
5) After the incubation period, the test stick is removed, and rinsed in cool water for 10 seconds.
6) The test stick is then placed into another labelled tube containing substrate solution.
7) After another 30 minute incubation period, the test stick is washed again in water to stop the color reaction.

When LH is present, enzymatic hydrolysis of the substrate to blue end product occurs in proportion to the amount of LH present in the sample.

D. **Assay Sensitivity and Detection Limit**

The OvuKit test is specific to intact LH and is sensitive to 20 mIU/mL. Clinical studies (Vermesh et al. 1987) demonstrated that this assay predicts ultrasound-detected ovulation more reliably (87.5%) than single daily serum LH (84%). While specific for LH, the assay is cross reactive with urinary hCG. Other homologous hormones, FSH and TSH, do not cross-react substantially. False positives from pregnancy (hCG) cross-reaction are obviated by pre-testing with the Tandem Icon II.

V. EFFECTS OF TEST CONDITIONS

A. **Simulated Conditions:** Patient samples and kit reagents were subjected to conditions that mimicked a day of testing in Tanzania. All samples, controls, and kits were allowed to reach room temperature before the manipulations began, and all tests were performed at room temperature. Each experiment was designed to test the breakdown of one aspect of the test while holding other variables constant.

Results showed that heating at 32°C for five hours had no effect on any aspect of the Tandem Icon II hCG assay. High temperatures stimulated the reaction in the OvuKit LH assay, such that the test results appeared ten minutes earlier with heated samples than the control. Heat appeared to have no effect on the actual results, however.

B. **Actual Conditions:** Kits and reagents were kept unrefrigerated, in a lined styrofoam box for at least 30 days (maximum, 45 days) before use. During this time,

ambient temperatures fluctuated from approximately 5°C to 32°C. Upon collection, urine samples were kept in the shade, unrefrigerated, for up to eight hours before testing. There was no discernable difference in test performance under field conditions from either ideal or simulated lab conditions. If hormone breakdown in samples did occur, this would increase the chance of a negative ascertainment.

VI. RESULTS

A. Pregnancy

116 Hadza women were tested for pregnancy using the Tandem Icon II assay; of these, 86 are of known age. Table 1 shows the prevalence of pregnancy for each ten year age category of all post-menarcheal women. Young women between the ages of 18 and 25 had the lowest pregnancy rate of all reproductively active women; the youngest woman pregnant in this study was 18.3 years old. By contrast, nearly 30% of all women between the ages of 35 and 45 were pregnant at the time of the study. Excluding women who have entered the menopausal transition (Table 2), 50% of women in this age category were pregnant. The oldest woman who tested positive for hCG was 45.9 years old.

B. Menopause

82 Hadza women were tested at least once for LH; of these, 68 are of known age. Table 3 demonstrates the prevalence of persistently elevated LH in these women. No woman under the age of thirty had consistently elevated LH levels; 18.2% of women under 40 showed patterns of elevated LH indicative of menopause. By age 45, over 60% of Hadza women had entered the menopausal transition. A probit using a maximum likelihood loss function was used to calculate median age at persistent LH elevation (inferred menopausal status), which was 41.9 years (Figure 6). Even if one uses a 1.5-year correction factor for premenopausal LH rises, the median age at menopause is 43.4 years. This is early: by contrast, the median age of menopause has been found to be 49.6 years for European women (Brand and Lehert 1978).

C. Completed Family Size

Based on our findings of menopausal status of individual Hadza women, we were able to determine the completed family size of 40 women. Through interview, we collected the total parity (both live and dead children) from 36 women, and the total live parity from 4 additional women. Hadza women gave birth to an average of 5.69 children (range 0-10), and by the end of their reproductive careers had an average of

3.4 surviving children (Table 4). This is slightly higher than the number of live births reported for the !Kung San, who average 4.7 live births per woman, with 40% of these dying before they reach maturity (Lee 1979). The overall fertility rate of the Hadza is, however, lower than the 6.6 births reported as the average for sub-Saharan African populations (Bongaarts, Frank and Lesthaeghe 1984).

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VII. CONCLUSIONS

These findings call for more in-depth investigations of fertility that use a broader range of quantitative physiologic measures, for such measures can reveal microprocesses shaping the female reproductive life cycle. We find the methods discussed here to be novel and useful additions to the field study of reproductive and behavioral ecology, as well as demography. Immediate ascertainment of the reproductive status of women can inform studies of behavior, as well as of demographic events. Analysis of data from applying these tests to the Hadza yielded these 3 preliminary findings:

1) Hadza accelerate their childbearing across the reproductive life span, so that pregnancy rates were lowest among younger and highest among older women.
2) Menopause (inferred from persistently elevated LH) occurs early among Hadza, at age 43.4 years.
3) Knowledge of menopausal status allows computation of completed family size from a larger database. At 4.7 births, Hadza fertility is rather strong (1 more than !Kung), despite the apparent early age at menopause.

Several confounds may limit the value of conclusions from this sample. They include small sample size, shallow time frame, lack of absolute ages (ranked ages were used), and effects of dry environment on urine concentration. The issue of possible early bias in menopausal age from our use of an LH criterion is currently under examination in a longitudinal study of menopausal transition.

also comment about unusual village setting

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age category	n	negative hCG	positive hCG	% pregnant
15.0-24.9	28	23	5	17.9
25.0-34.9	19	15	4	21
35.0-44.9	17	12	5	29.4
45.0-54.9	10	10	0	0
55.0-59.9	12	12	0	0
TOTAL	86	72	14	16.3

Prevalence of Pregnancy in Hadza Women

TABLE 1

age category	n	negative hCG	positive hCG	% pregnant
15.0-24.9	28	23	5	17.9
25.0-34.9	17	13	4	23.5
35.0-44.9	10	5	5	50
45.0-54.9	2	2	0	0
55.0-59.9	1	1	0	0
TOTAL	58	44	14	24.1

Prevalence of Pregnancy in Premenopausal Hadza Women

TABLE 2

age category	n	negative LH	positive LH	% elevated LH
20.0-29.9	9	9	0	0
30.0-39.9	22	18	4	18.2
40.0-49.9	13	4	9	69.2
50.0-59.9	11	1	10	90.9
60.0-69.9	13	0	13	100
TOTAL	68	32	36	53

Prevalence of Elevated LH in Hadza Women

TABLE 3

	ranked age of women	total parity	live parity
n	35	36	40
minimum	33.4	0	0
maximum	78.4	10	6
mean	54.9	5.69	3.4
SD	12.2	2.16	1.63

Completed Family Size of Hadza Women

TABLE 4

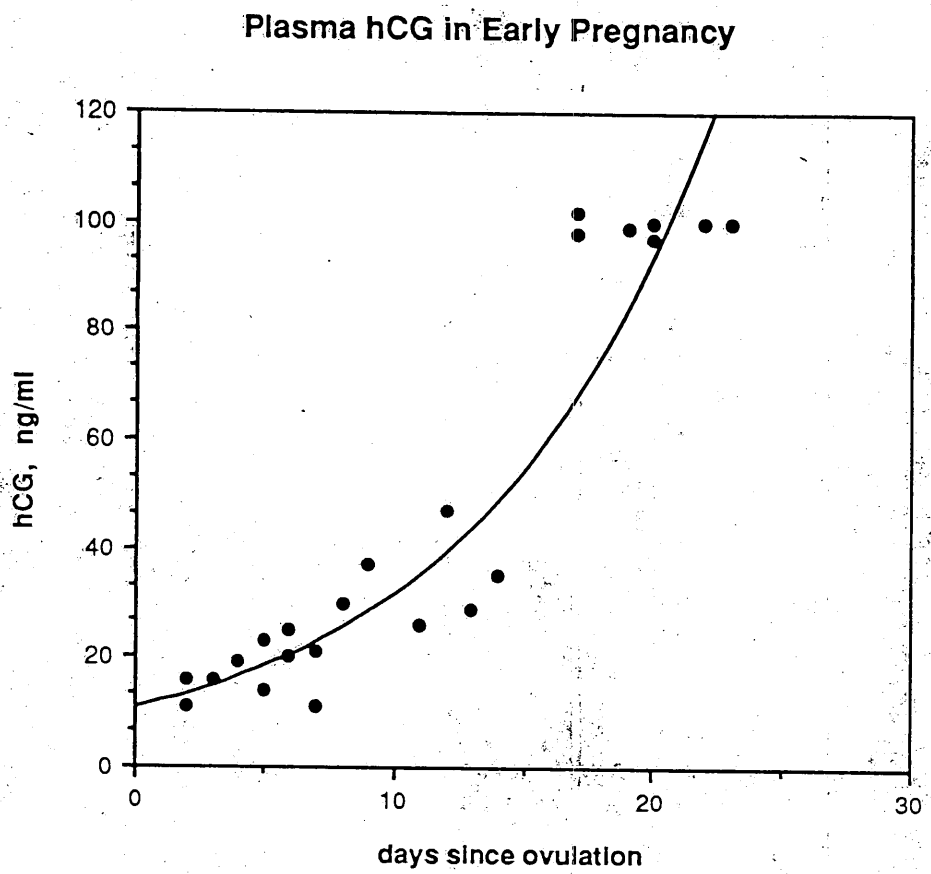
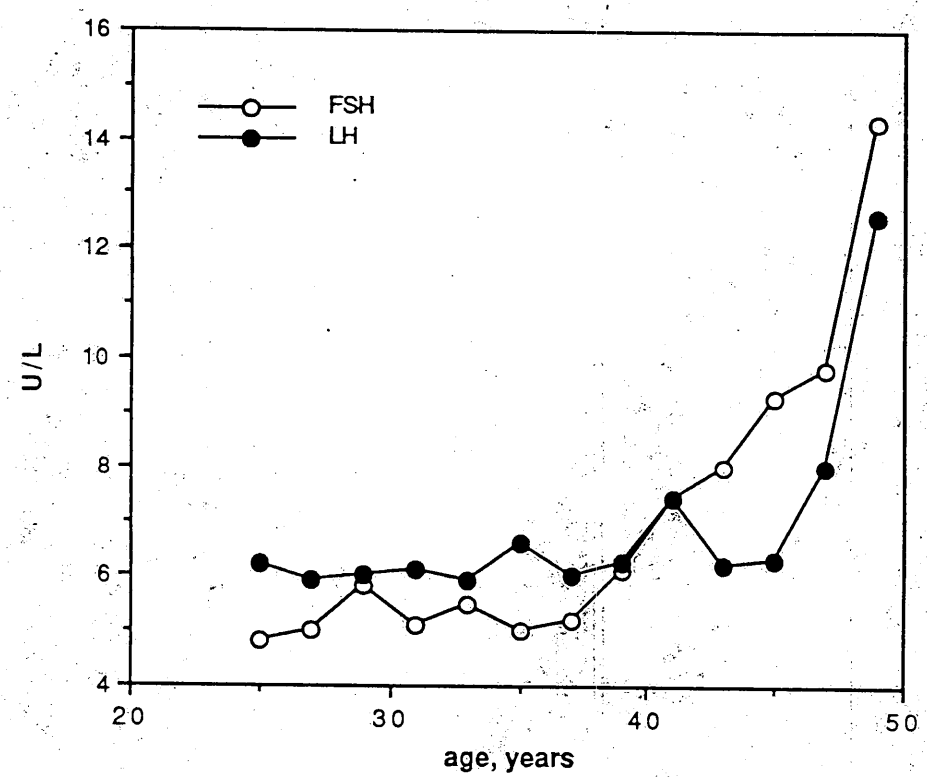


FIGURE 1

Redrawn from: R.G. Edwards (1980) Conception in the Human Female

Serum Gonadotropin Changes with Age in Women



from: Lenton, E.A., et al. (1988) Maturitas 10:35-43

FIGURE 2

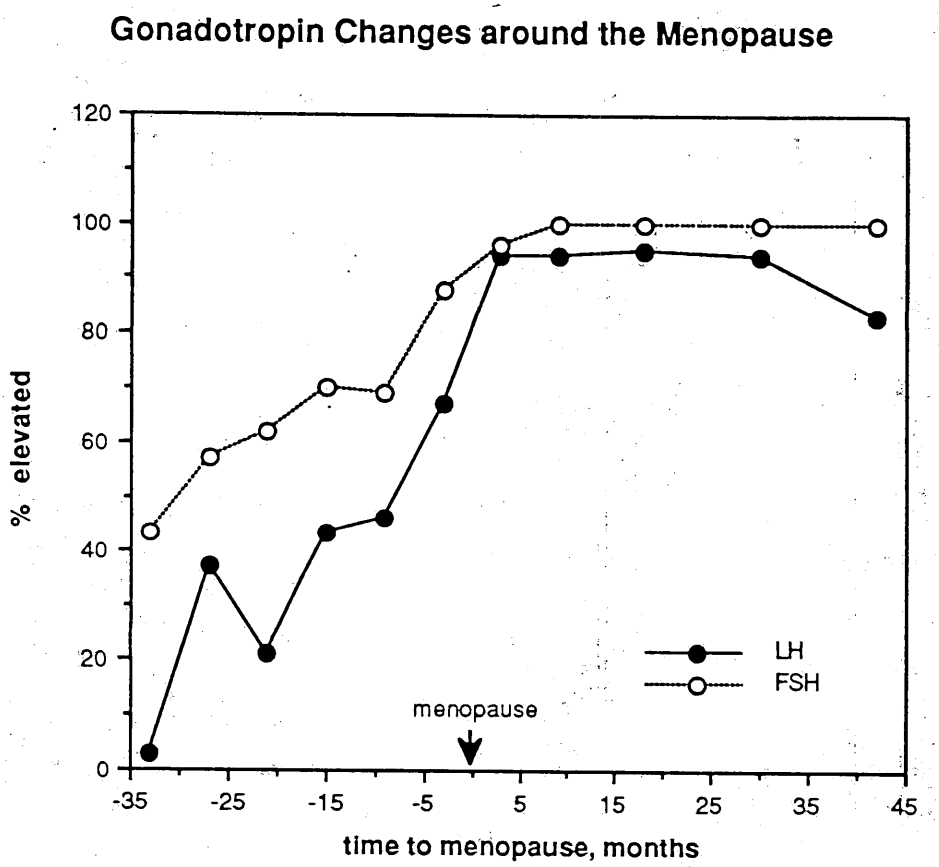


FIGURE 3

Redrawn from: Rannevik, C., et al. (1986) Maturitas 8:297-307.

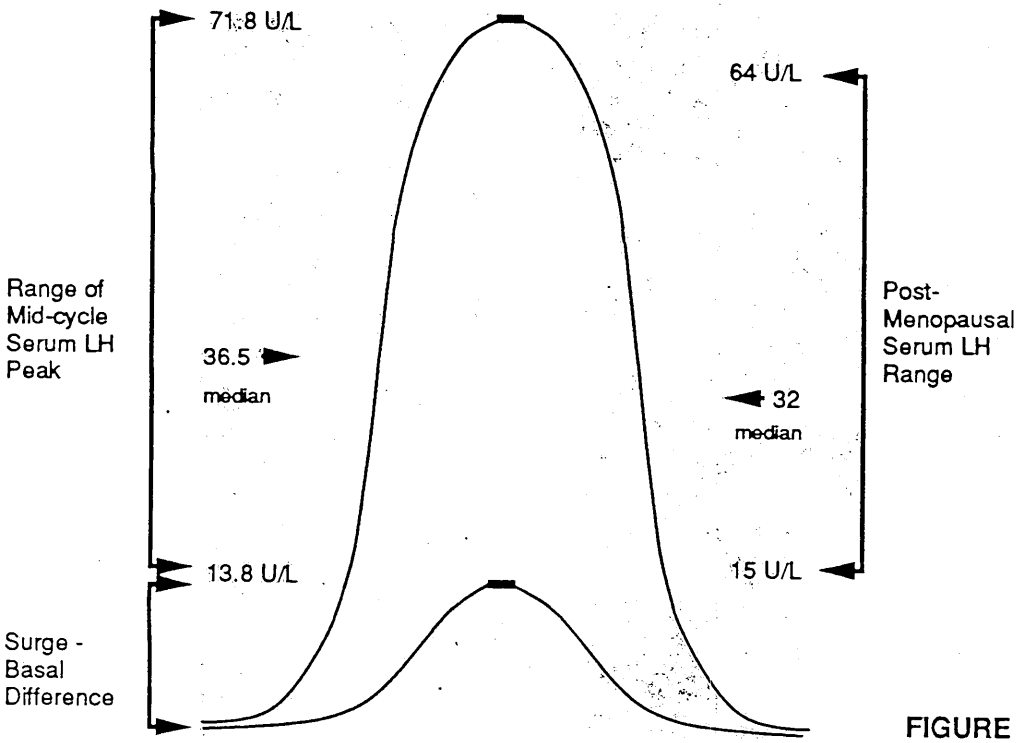


FIGURE 4

Comparison of Serum LH Concentrations

during the Mid-Cycle Peak and after Menopause

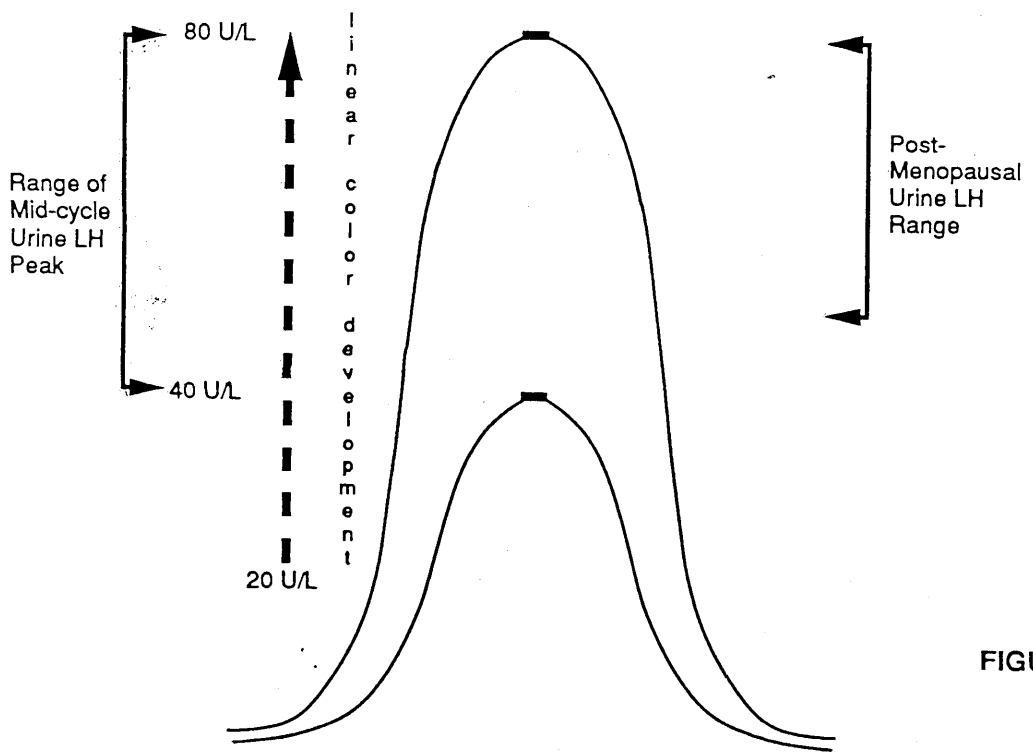
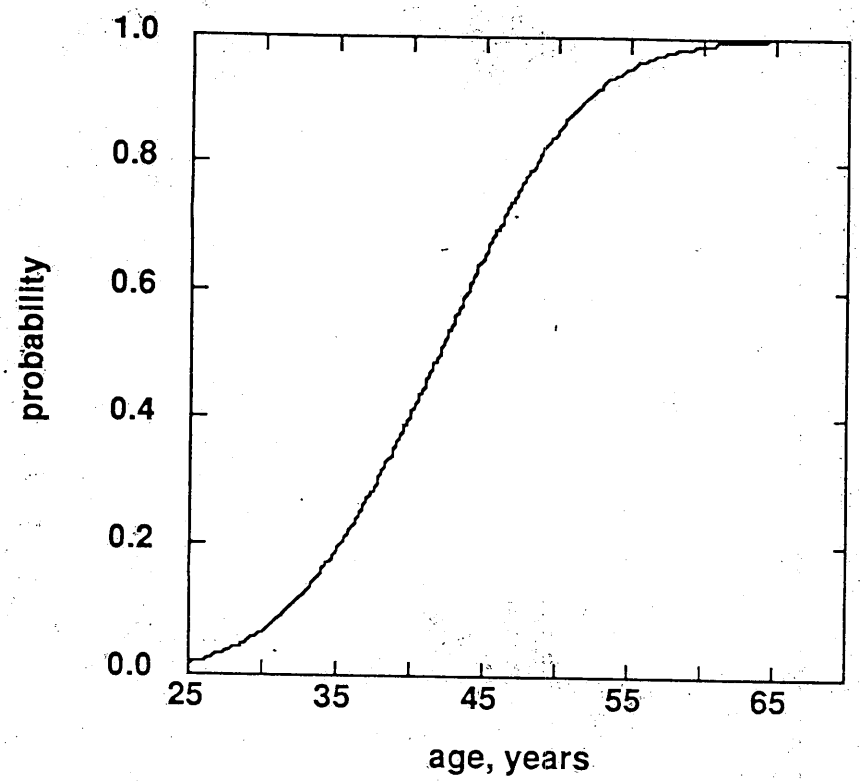


FIGURE 5

Comparison of Urinary LH Concentrations at the Preovulatory Peak

and after Menopause with Range of Linear OvuKit Color Reaction



Probability of Persistently Elevated LH in Hadza Women

Figure 6