

# Intensified Rates of Venous Sampling Unmask the Presence of Spontaneous, High-Frequency Pulsations of Luteinizing Hormone in Man\*

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**ABSTRACT.** To test the validity of venous sampling rates that are generally used to characterize pulsatile LH release in man (e.g. sampling every 15–20 min), we characterized apparent LH pulse frequency in blood withdrawn variously at 20- or 4-min intervals in 19 men, at 2-min intervals in 14 men, and at 1-min intervals in 6 men. In an effort to minimize detection bias, significant LH pulses were evaluated objectively using a computerized pulse-detection algorithm, which tended to maximize recognition of true-positive LH pulses, and minimize false-positive and false-negative pulses. Under these conditions, intensified rates of venous sampling at 4-, 2-, and 1-min intervals exposed approximately 3.6, 4.9, and 13.7-fold more LH pulses, respectively, than could be discerned at a 20-min sampling frequency. In addition, more rapid rates of venous sampling disclosed a previously unobserved pattern of LH pulses, in which

higher frequency, lower amplitude LH pulsations were interposed among low frequency, high amplitude LH peaks. Quantitatively, LH pulses unmasked by intensified rates of venous sampling exhibited significantly lower pulse amplitudes, expressed either as a fractional (%) or absolute (mIU/ml) increment, than pulses identified at 20-min intervals.

In conclusion, we demonstrated that intensified rates of venous sampling unmask a significant number of otherwise unrecognized LH pulses in the circulation of normal men. Moreover, because generally employed sampling rates overlooked these more rapid physiological fluctuations in LH concentrations, patterns of both high and low frequency LH pulsations must now be characterized in various states of health and disease using more rapid sampling paradigms. (*J Clin Endocrinol Metab* 59: 96, 1984)

**D**ISTINCTLY episodic fluctuations in circulating concentrations of LH reflect intermittent stimulation of pituitary gonadotropin release by pulses of hypothalamic GnRH (1–3). This pulsatile pattern of brain GnRH release is in turn controlled by, and effectively integrates, multiple input from central neural mechanisms (4–7). The regulation and operation of these central neural mechanisms in the human cannot be examined directly. Moreover, neural mechanisms in man cannot be investigated by estimates of peripheral venous

levels of GnRH, because at present such measures do not reliably quantitate GnRH secretion into the hypothalamo-pituitary portal circulation. Thus, as an alternative probe of intermittent GnRH release, investigators have characterized patterns of peripheral LH pulsations under diverse conditions of health and disease (8–15). This strategem requires that venous sampling rates be sufficiently rapid to identify the majority of physiological LH pulsations that actually occur.

Typical characterizations of LH pulse patterns involved repetitive venous sampling at 15 to 20-min intervals to provide a series of LH concentrations for pulse analysis (16–18). A pivotal assumption of any such analysis is that the sampling rate chosen is sufficiently frequent to embrace or capture all but a negligible portion of LH pulses truly present. The validity of this critical premise has never been tested systematically. In addition, the corollary assumption that different sampling rates lead to similar inferences regarding the configuration or pattern of observed LH pulses has not been

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examined.

In the present work, we investigated the impact of sampling rate on the number and the character of LH pulses in normal men.

## Subjects and Methods

### Subjects

Thirty three normal men aged 21–30 participated in this study after providing written informed consent. The study was approved by the Human Investigation Committee of the University of Virginia. Each man had a normal history and physical examination, including normal external genitalia and testis volumes. In addition, serum concentrations of the following hormones were within the normal range: testosterone, estradiol, free T<sub>4</sub>, PRL, LH, and FSH.

### Procedures

The men were admitted to the Clinical Research Center and an indwelling heparin-lock needle was inserted into a forearm vein at least 1 h before blood sampling. Blood was removed at 1-min intervals for 2 h in 6 men, at 2-min intervals for 2 h in 8 other men, at 4-min intervals for 8 h in 5 men, and at 20-min intervals for 8 h in 14 other men. The volunteers were kept fasting and supine for the sampling sessions, which lasted for 2 or 8 h, as noted above.

### Sampling procedures and RIA

Samples were allowed to clot at room temperature, and the serum was stored at –20 C before assay. All samples from an individual were analyzed in the same assay to eliminate inter-assay variability. LH concentrations were assayed in triplicate using a modification of the method of Odell *et al.* (19). The reagents were those described previously (20).

To define the interassay variability precisely at multiple points along the displacement curve, 7 pools of serum were assayed 9 times each. The serum pools spanned the useful range of LH values in normal men. In addition, to provide a more precise estimate of within-assay variance, 7 other pools of normal adult male serum were assayed as 90–360 replicates in 6 separate assays. These larger series provided control serum pools with which to assess the performance of the pulse detection algorithms. The intraassay coefficients of variation computed from all serum pools ranged between 5.5% and 10.1% for the levels of LH measured in the normal men in the present study, specifically, 5.5%, 6.5%, 8.0%, and 10.1% for LH concentrations of 10 mIU/ml, 6 mIU/ml, 4 mIU/ml, and 2.5 mIU/ml, respectively.

### Quantitative analysis of pulsatile hormone secretion

Pulsatile patterns of LH release were analyzed by a modification of the method of Santen and Bardin (16), using the Control Data Corporation Cyber 720 computer. The original algorithm of Santen and Bardin identified a pulse as a 20% or greater increase in LH concentration above the preceding nadir. In these investigators' laboratory, the criterion of 20% repre-

sented approximately 3 times the intraassay coefficient of variation. Instead of using a fixed 20% threshold, we modified this algorithm to permit detection of pulses that exceeded our relevant within-assay coefficients of variation by 4 times (or, in some designated figures, by another multiple). The relevant within-assay coefficient of variation for any 1 subject was computed from 30–90 assay replicates of a pool of serum derived from that individual's samples and run in the same assay.

To evaluate the performance of our pulse enumeration method, serial LH values from the seven distinct serum pools (control series) were also analyzed for the presence of (spurious) LH pulses.

### Statistical analyses

Data are presented as means  $\pm$  SEM. Analysis of variance (F test) was employed to estimate the effect of sampling frequency on the results (21). Significance was construed for  $P \leq 0.05$ .

### Simulation of less frequent sampling intervals

Data derived from 1-, 2-, or 4-min sampling intervals were also analyzed by omitting successive samples in order to permit analyses of values collected at less frequent intervals. For example, deleting every other point in the serial 1-min values provided a series of samples actually collected at 2-min intervals.

## Results

### *Influence of procedural (intraassay) error on pulse detection*

To test the numerical validity of our pulse analysis algorithm, we analyzed a total of 335 LH values derived from control pools of male serum for the presence of spurious pulses. Seven control serum pools were chosen to encompass the normal range of serum LH values in men, and had a mean value of 4.78 mIU/ml (range, 2.78–12.4 mIU/ml). Each serum pool yielded at least 30 mean (of triplicate) observations. Two pools provided 120 mean LH values. The serial LH values derived from these pools, and the sequential LH values derived from volunteers undergoing repetitive venous sampling, were analyzed for apparent LH pulses using increasingly stringent threshold criteria.

Thresholds for pulse detection in the Santen and Bardin program were set at 2, 3, 4, 5, . . . 20 times the pertinent intraassay coefficient of variation. Figure 1 depicts the results of this analysis applied to control serum pools and to samples collected at 20-min (*panel A*) or 4-min intervals (*panel B*) in the same 5 men, and at 1-min intervals in 6 other men (*panel C*). We identified false-positive LH pulses (shown by the interrupted curves) as those enumerated in the control noise pools, which comprised the same number of samples as the volunteer series. As the threshold criterion was increased

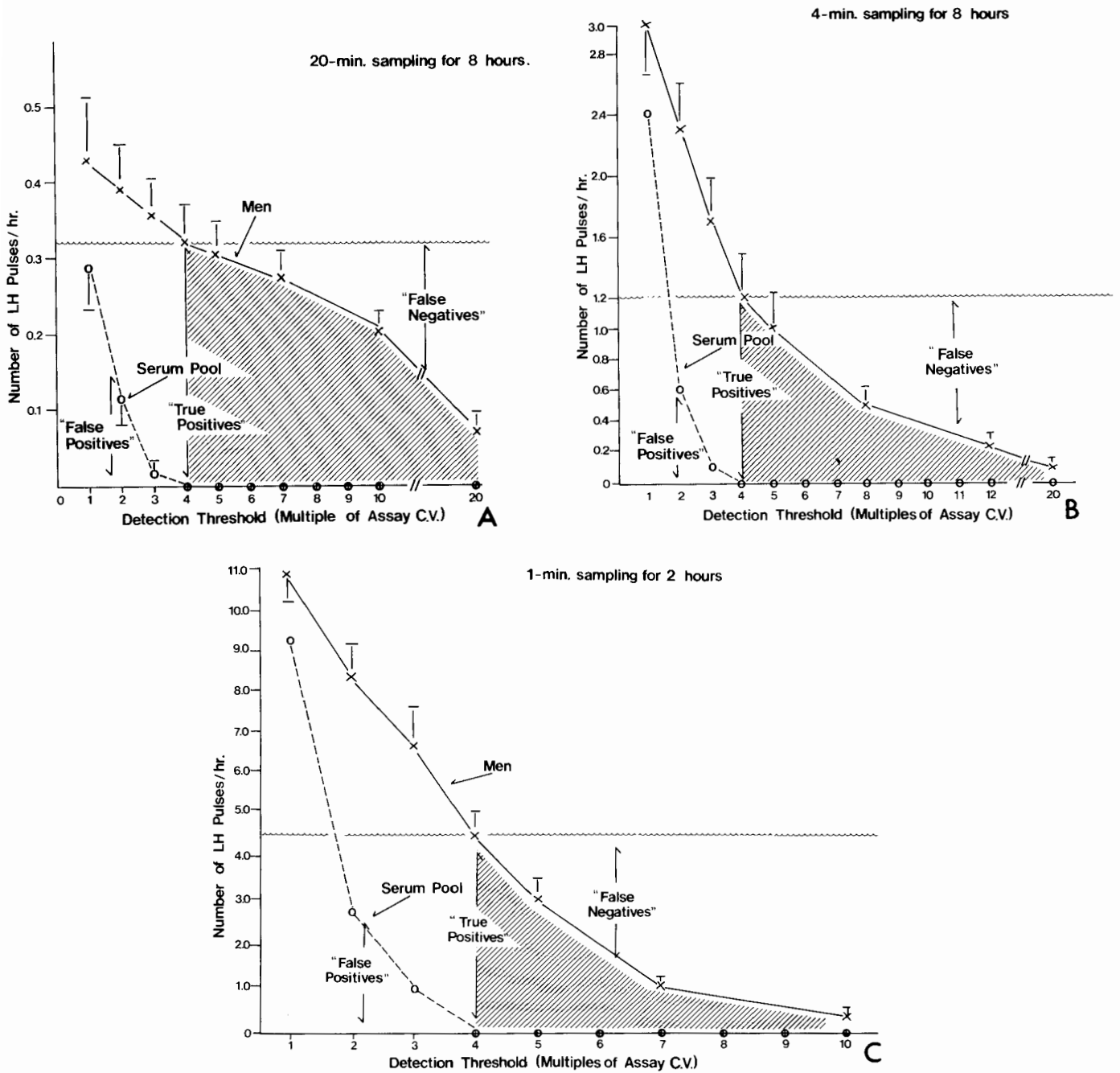


FIG. 1. Influence of the detection threshold on apparent LH pulse frequency. The number of LH pulses identified per sampling series (8 or 2 h) is plotted against increasingly stringent detection thresholds (expressed as integral multiples of the intraassay coefficient of variation). In each panel, the upper solid curve depicts the apparent pulse frequency for the normal men, and the lower interrupted curve gives the number of spurious LH pulses found in control serum pools comprising the same number of samples. The influence of the detection threshold on apparent LH pulse frequency was analyzed at sampling rates of 20 min (A), 4 min (B), and 1 min (C). In each case, imposing higher threshold requirements progressively reduced the number of LH pulses in both the normal men (upper curve, Men) and in the control-serum pools. At a threshold criterion of 4 times the intraassay coefficient of variation, the frequency of false-positive LH pulses in the serum pool decreased to less than 0.5%. Thus at this and higher thresholds, LH peaks detected in the experimental series provide some estimate of true-positive LH pulses (cross-hatched region). However, for more stringent thresholds, true LH peaks are more likely to be missed. We have denoted this tendency by the false-negative region of the curve. Data are means  $\pm$  SD ( $n = 5$  men in A and B;  $n = 6$ men in C).

progressively, fewer false-positive LH pulses were detected in the control serum pools. When a criterion of 4

times the intraassay coefficient of variation was applied, false-positive LH pulses occurred at a rate of less than

0.5% in the control serum pools. However, at lower thresholds (e.g. 2 or 3 times the intraassay coefficient of variation), estimates of LH pulse frequency were significantly contaminated by spurious pulses. Conversely, at very high detection thresholds (e.g. 5- to 20-fold the intraassay coefficient of variation), there was an increasing likelihood of presumptively false-negative observations with a consequent underestimation of pulse frequency. Thus, for the purposes of the present study, we used a threshold criterion of 4 times the intraassay coefficient of variation to provide an estimate of true-positive LH pulses (denoted by the shaded region of the curve).

#### *Influence of sampling rate on the detection of LH pulses*

**Influence on apparent LH pulse frequency.** The results in Fig. 2 demonstrate the profound ( $P < 0.001$ ) effect of increased rates of venous sampling on the detection of LH pulses in normal men during resting conditions. In particular, sampling at 20-min intervals disclosed  $0.32 \pm 0.03$  LH pulses/h (mean  $\pm$  SEM in 19 different men), but more rapid sampling at 4-, 2-, or 1-min intervals revealed  $1.17 \pm 0.20$ ,  $1.57 \pm 0.30$ , and  $4.41 \pm 0.60$  LH pulses/h (in 19, 14, and 6 men, respectively). Since these estimates were obtained under conditions in which the contribution of intraassay noise was substantially reduced by the high threshold chosen, we infer that the sampling interval *per se* markedly influences the detectability of LH pulses in man. Moreover, a highly significant influence of the

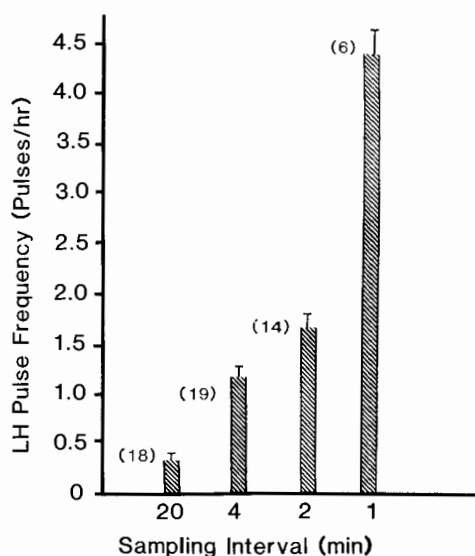


FIG. 2. Critical influence of the sampling interval upon the estimate of LH pulse frequency. Blood samples were drawn at 20-, 4-, 2-, or 1-min intervals in normal men. The subsequent individual LH series were analyzed for significant LH pulses (see *Subjects and Methods*). The number of LH pulses detected per hour is given on the vertical axis. The number of men studied is given in parentheses beside the corresponding bar graph. Data are means  $\pm$  SEM.

sampling interval on the detection of LH pulses could also be demonstrated when our results were analyzed with 2 other independent pulse-detection algorithms (17, 18) (Veldhuis *et al.* *Am J Physiol*, submitted for publication).

A typical example of the changes in LH pulse frequency for 20-min 4-min sampling intervals in one man is shown in Fig. 3. This figure illustrates that high frequency, low amplitude LH pulsations were exposed when the sampling interval is reduced from 20 to 4 min. Similarly as illustrated for another subject in Fig. 4, further reduction of the sampling interval from 4 min to 1 min unmasked a significant number of LH pulses not otherwise demonstrable.

**Influence on LH pulse amplitude.** As summarized in Table 1, shortening the venous sampling interval was associated with the detection of LH pulses of signifi-

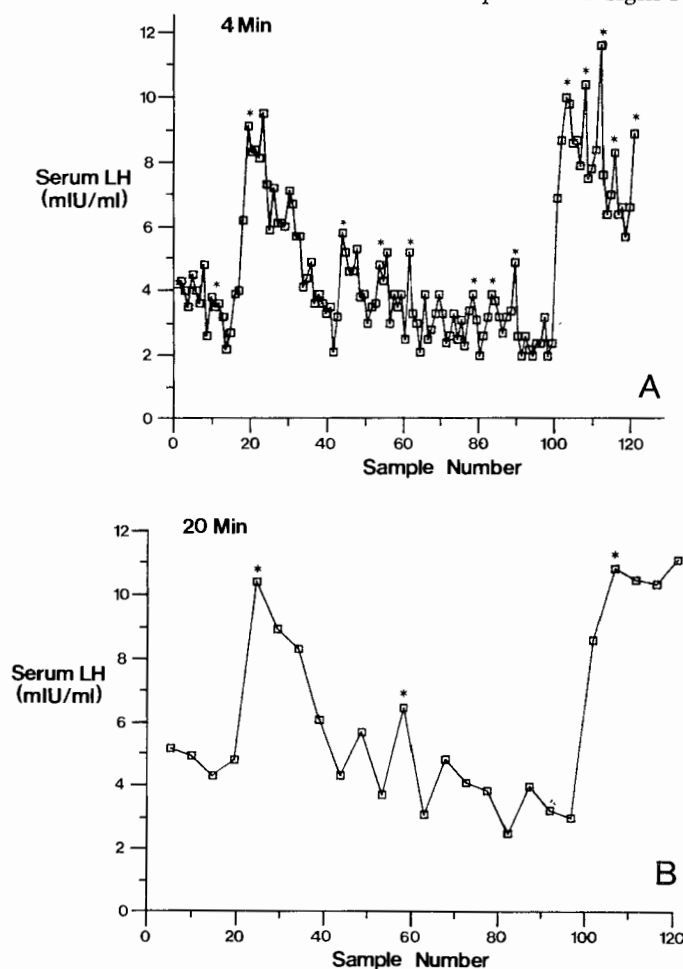


FIG. 3. Impact of varying the sampling interval on the profile of episodic LH release in a normal man. Blood was withdrawn at 4-min intervals for 8 h yielding serial LH values for pulse analyses (A). Intervening samples were omitted to permit pulse analyses at a conventional sampling rate of every 20 min (B). The vertical axis gives the serum LH concentration in mIU/ml, and the horizontal axis shows the serial LH sample number. Individual pulses are denoted by \*.

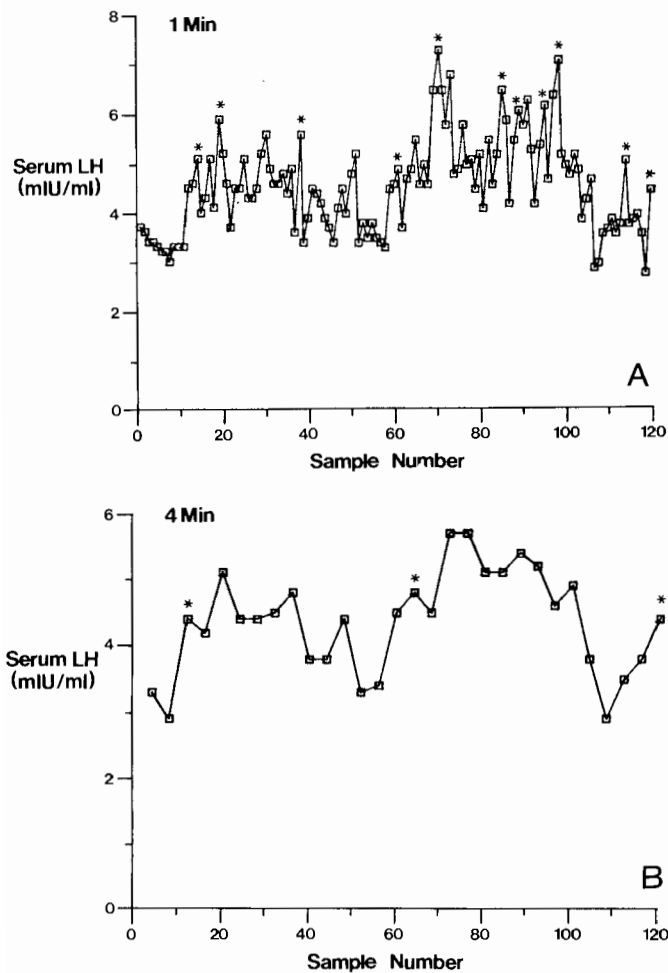


FIG. 4. Disclosure of high frequency LH pulses by intensified rates of venous sampling. Blood was withdrawn at 1-min intervals for 2 h from a normal man. Serial samples denoted on the horizontal axis were analyzed for their content of LH in mIU/ml given on the vertical axis. Significant individual LH pulses are identified by \*. A and B depict results from the 1- and 4-min sampling, respectively, in this subject. The 4-min data were derived by omitting their successive samples from the original 1-min series.

TABLE 1. LH pulse amplitude characteristics in relation to venous sampling interval

Sampling intervals (min)	Fractional (%)	Increment (mIU/ml)
20 (n = 19)	131 ± 9	4.66 ± 0.65
4 (n = 19)	77 ± 6	3.50 ± 0.41
2 (n = 14)	67 ± 7	3.15 ± 0.31
1 (n = 6)	59 ± 3	2.84 ± 0.30

Intervals (min) at which serial blood samples were obtained. Data are means ± SEM (n designates the number of individual men studied at each sampling rate.)

cantly lower mean amplitude. Both the mean fractional (% above nadir) and mean incremental (mIU/ml above nadir) amplitude were significantly lower at higher rates of sampling. These data provide a numerical basis for

the visual impression that high frequency, lower amplitude LH pulses are superimposed upon and interspersed between higher amplitude, less frequent LH pulses (Figs. 3 and 4).

*Internal validity of the pulse frequency estimates.* As one probe of the internal consistency of the pulse-frequency estimates, we have analyzed the rapidly sampled series in three additional ways:

1) For the 1-min series drawn over 2 h in 6 men, we analyzed LH pulse frequency separately in the even-numbered (2, 4, 6, . . . 120 min) samples and the odd numbered (1, 3, 5, . . . 119 min) samples. This approach yields individually processed samples drawn at 2-min intervals. The independent pulse frequency estimates were  $5.17 \pm 0.70$  pulses/2 h for the odd and  $5.33 \pm 0.95$  pulses/2 h for the even numbered series.

2) The 1-min series was analyzed for its 4-min constituents, beginning either with the 0-, 1-, 2-, or 3-min sample, which yielded independent pulse frequency estimates of  $3.5 \pm 0.5$ ,  $3.4 \pm 0.7$ ,  $3.5 \pm 0.5$ , and  $3.3 \pm 0.6$  pulses/2 h.

3) The 4-min series was analyzed for its 20-min components, beginning either with the 0-, 4, 8, 12, or 16-min samples, which yielded independent pulse frequency estimates of  $3.7 \pm 0.7$ ,  $3.8 \pm 0.8$ ,  $3.6 \pm 0.8$ ,  $3.4 \pm 0.5$ , and  $3.8 \pm 0.4$  LH pulses/8 h.

These analyses effectively include variability introduced by individual sample processing (e.g. clotting, centrifugation, sample transfer, freezing, and thawing), as well as intraassay measurement error, and hence serve to corroborate the validity of our results. In addition, when threshold criteria of either 3 or 5 times the intraassay coefficients of variation were used to analyze the LH series, there was still a highly significant relationship between apparent LH pulse frequency and sampling intensity ( $P < 0.001$ ). This increase occurred despite corresponding changes in relative false-positive (type I) and false-negative (type II) error rates for these less or more rigorous criteria.

## Discussion

The present investigations demonstrate that intensified rates of venous sampling reveal previously unrecognized spontaneous patterns of relatively high frequency and low amplitude LH pulsations during resting physiological conditions in normal men. For example, frequent venous sampling at 4-min intervals disclosed 3.6-fold more LH pulses than were found at a sampling rate of every 20 min. Further abbreviation of the venous sampling interval to 2 or 1 min exposed, respectively, 4.9 and 13.7 times more LH pulses per unit time than could be recognized at 20-min sampling rates.

Visual inspection of the LH profiles obtained at more

rapid sampling rates revealed relatively lower amplitude and higher frequency LH pulses that were superimposed upon (and interspersed among) large LH peaks, as typically defined at 15- to 20-min sampling intervals. This pattern was expressed numerically in significantly lower fractional (%) and incremental (mIU/ml) LH pulse amplitudes demonstrable at more frequent sampling rates.

The disclosure of this previously unrecognized pattern of LH pulses was not readily attributable to artifact or bias in the processing or RIA of blood samples, the pulse detection algorithms, or the selection of subjects, in view of the following considerations. First, the threshold criterion of 4 times the individual intraassay coefficient of variation we used in the pulse-detection algorithm was adjusted to reduce the false-positive (type I) error rate to less than 0.5%. Since increasingly rigorous threshold criteria result in more false-negative (type II) errors, use of this somewhat conservative threshold permits us to err, if anything, on the side of underestimating the increase in LH pulse frequency at more rapid sampling rates. Thus, when less stringent detection criteria (*e.g.* 3-fold the intraassay coefficient of variation) were employed, there was an even greater increase in apparent LH pulse frequency at more rapid sampling rates. Such an increase would reflect in part the influence of a greater type I error and a lesser type II error. Conversely, when more rigorous criteria (*e.g.* 5-fold the intraassay coefficient of variation) were used, there was still a major effect of sampling intensity on apparent LH pulse frequency despite a decrease in the relative type I/ type II error rates. Moreover, using two other independent methods for pulse enumeration (17, 18), the LH pulse frequency estimates also were highly dependent upon sampling rates. Second, estimates of LH pulse frequency at more rapid rates of venous sampling were relatively consistent internally, when separate subsets of individually processed samples were compared in several groups of men. Third, the significant increase in LH pulse frequency observed at shortened sampling intervals is in accord with classical sampling theory, which predicts that increased sampling rates would be required to disclose the presence of high frequency pulses (22). And, fourth, compared to 20-min sampling rates, more LH pulses were found at 4-min sampling intervals in 19 men and at 2-min sampling intervals in 14 men. Results from this large group of subjects suggest the generality of our observations. Nonetheless, we recognize that the even more striking increase in apparent LH pulse frequency at 1-min sampling rates may reflect in part the influence of increased pulse detection as well as greater intrinsic variability introduced by sampling at these extremely rapid rates, and the small number ( $N=6$ ) of subjects.

In view of these collective considerations, we believe that relatively high frequency, low amplitude LH pulses

occur spontaneously in normal men, and that their recognition requires rapid rates of venous sampling. This inference in normal men is in accord with observations of Medina *et al.* (23) of short term LH fluctuations in three postmenopausal women, those of Leppaluoto *et al.* (24) in four men and one woman sampled at 4-min intervals for 80 min, and those of Filicori *et al.* (25) who sampled at 5- and 10-min intervals. Unfortunately, these initial reports cannot be readily compared with the present work, because they either included quite different physiological states, studied very few subjects, did not compare sampling rates within the same subject, sampled for a shorter period, or importantly did not adjust pulse-detection criteria to minimize false-positive errors associated with enumerating pulses in large numbers of samples. We patterned our concept of modifying the threshold criterion to limit false-positive (type I) errors after that of Ross *et al.* (26), and for this purpose selected a somewhat more conservative criterion. Since there is presently no means available to define absolutely the presence of true-positive LH pulses, an exact type II (false-negative) error rate cannot be ascertained. Thus, our results provide a close estimate but not an absolute value for the increase in LH pulse detection afforded at more rapid sampling rates.

Rapid (every 2.5 or 4 min) venous sampling in experimental animals also recently disclosed patterns of higher frequency LH release akin to that found in the present studies in normal men. In particular, sampling at 4-min intervals in the follicular phase of the sheep (27) or at 2.5-min intervals in the orchietomized rat (28) yielded LH pulse frequencies of approximately 1.8 pulses/h and 3 pulses/h, respectively. The results in these species compare with 1.6 pulses/h on average estimated in our normal men sampled at 2-min intervals. Most importantly, these results in the human, sheep, and rat suggest that high frequency, low amplitude LH release may be a general physiological property of the hypothalamo-pituitary interface, in which very rapidly occurring neural events impinge upon, and are integrated by, a responsive endocrine system.

Based upon analyses of peripheral LH pulses enumerated at rapid sampling rates (present study, 27, 28), the mammalian pituitary gland seems to be responsive to both high and low frequency GnRH signals. The exact physiological impact of rapid lower amplitude GnRH signalling at the pituitary level is not known, but pituitary secretory responsiveness is clearly strongly modulated by the frequency component of the GnRH stimulus (29-32). This mode of signalling does not appear to desensitize the pituitary gland but may modulate the informational content of the GnRH and/or subsequent LH pulse signal at the level of the respective target glands. Further studies in man and other species will

ultimately be required to help clarify whether the high frequency and lower amplitude LH pulse signal principally reflects proximate events at the hypothalamic-pituitary level, or also exerts a physiological impact on gonadal target cells.

In summary, intensified rates of venous sampling in normal men revealed spontaneous, high frequency LH pulsations that were not demonstrable at less rapid sampling rates, presumably in part because of their relatively low amplitude characteristics. The recognition that such high frequency LH pulses occur under physiological conditions in normal men creates a need to define further patterns of high and low frequency LH pulsations in various states of health and disease.

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