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# A CYTOCHEMICAL BIOASSAY METHOD FOR THE DETERMINATION OF LUTEINIZING HORMONE IN BIOLOGICAL FLUIDS AND TISSUES

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**1** The ability of luteinizing hormone (LH) to alter the reducing activity of corpora lutea in rat ovarian sections has been exploited to develop a new cytochemical bioassay for the hormone.

**2** Sections of ovaries, removed from mature rats during the second day of dioestrus, were incubated with either standard LH or samples diluted for assay, stained immediately for reducing potential and the intensity of the stain measured by scanning and integrating microdensitometry.

**3** An inverse linear relationship existed between the density of the stain and the logarithm of the concentration of standard LH (68/40) and the dose-response lines of serial dilutions of rat or human plasma were parallel with those of the standard.

**4** The method was found to be accurate, specific, sensitive, precise and suitable for the determination of LH in the rat and in man.

## Introduction

The abilities of luteinizing hormone (LH) and corticotrophin (ACTH) to reduce the concentration of ascorbic acid in the ovary (Parlow, 1958) and adrenal cortex (Sayers, Sayers & Woodbury, 1948) respectively have been made the basis of bioassay methods for their determination. Although these methods are accurate and specific they lack precision and sensitivity. With the introduction of cytochemical techniques for the measurement of the ascorbic acid changes it became possible to detect and estimate, with precision, low concentrations of the trophic hormones using small volumes of blood. However, the number of samples which could be conveniently assayed for LH or ACTH was severely limited because of the use of segments of ovarian (Rees, Holdaway, Kramer, McNeilly & Chard, 1973) or adrenal (Chayen, Loveridge, & Daly, 1972) tissue. The practicability of the method for the assay of ACTH was enhanced by the introduction (Alaghband-Zadeh, Daly, Bitensky & Chayen, 1974) of a modification using adrenal sections instead of segments which greatly increased the number of samples that could be assayed. This paper describes a cytochemical section method for the determination of LH in blood and pituitary tissue. Preliminary accounts of the method have been presented at a meeting of the Society for Endocrinology (Buckingham, Chayen, Hodges, Robertson & Weiss, 1979a) and at the XII Acta Endocrinologica Congress (Buckingham, Chayen, Hodges, Robertson & Weiss, 1979b).

## Methods

### *Animals*

Mature female Sprague-Dawley rats (Charles River, SPF) weighing 200 to 250 g were maintained at a constant temperature of 22°C for at least 2 weeks before use. Food and water were available *ad libitum*. Vaginal smears were taken daily and only those rats which had at least two regular 4-day oestrous cycles were used.

### *Luteinizing hormone standard preparation*

Luteinizing hormone standard preparation WHO 68/40 (Storring, Bangham, Cotes, Gaines & Jeffcoate, 1978) of which the potency was taken as 77 iu/ampoule, was dissolved in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.25% human serum albumin (Sigma) to prepare a solution, containing 1 iu LH/ml, of which aliquots were snap frozen in plastic tubes and stored at −70°C until required.

### *Assay procedure*

Rats were killed by cervical dislocation during the second day of dioestrus, the ovaries were removed rapidly and dissected free from periovarian tissue. The ovaries were placed in a Trowell's culture chamber and primed with LH ($10^{-7}$ i.u/ml in

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Trowell's T8 medium) for 4 min at 37°C. Priming with LH prevents the spontaneous ascorbate loss which sometimes occurs during the subsequent incubation of sections of the ovarian tissue. They were chilled immediately to −70°C by immersion in *n*-hexane for 45 to 60 s and transferred to chilled Pyrex tubes which were stored on dry ice. Within 48 h, serial cryostat sections (20 μm) were cut from each ovarian block and stored in the cryostat for not longer than 30 min. The sections were immersed simultaneously for 80 s in solutions at 37°C of either standard LH or samples diluted for assay (in Trowell's T8 medium containing $10^{-3}$M sodium ascorbate (Sigma) and 5% polypeptide (Sigma 5115) at pH 7.6). They were stained immediately for reducing potential as described by Alaghband-Zadeh, Daly, Tunbridge, Loveridge & Chayen (1973), mounted in D.P.X. mountant (BDH) and stored in the dark at room temperature. The density of the stain in a corpus luteum was measured at 660 nm by scanning and integrating microdensitometry. Ten readings were taken from each section.

*Blood samples*

Blood was collected, between 09 h 00 min and 10 h 00 min, from the trunks of rats after decapitation and from other species by venepuncture, into heparinized plastic tubes which were centrifuged immediately at $1.875 \times 10^3 g$ for 5 min. The plasma was separated and stored at −20°C for not longer than 1 month.

*Pituitary extracts*

Anterior pituitary glands removed from female rats killed by decapitation between 09 h 00 min and 10 h 00 min were homogenized separately in 1.0 ml 0.1 M HCl. The homogenates were transferred to a refrigerator (4°C) for 24 h and then stored at −20°C.

*Hormone preparations*

The following were used:- luteinizing hormone (WHO 68/40), follicle stimulating hormone (FSH, WHO 77/532), thyrotrophin (TSH, WHO 63/14), corticotrophin (WHO 74/583S), prolactin (WHO 2nd International Standard), LH α- and β-subunits (WHO 72/20 and 71/342), human chorionic gonadotrophin (HCG, WHO 75/537), HCG α- and β-subunits (WHO 75/569 and 75/551), lysine vasopressin (Sigma) and angiotensin II (MRC Blood Pressure Unit, Glasgow). Information on the chemical purity, etc. of the peptide preparations which were donated by the World Health Organisation is obtainable from the National Institute of Biological Standards and Control, London NW3. Corticosterone, oestradiol, testosterone and progesterone were donated by Organon Laboratories Ltd.

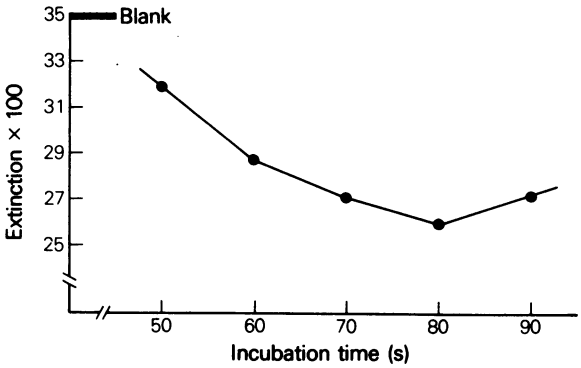


**Figure 1** The effect of variations in incubation time on the response (stain intensity expressed as integrated extinction) of ovarian sections to LH ($10^{-4}$ iu/ml). Every point is the mean of 4 determinations none of which differed from the mean by more than 10%.

The peptides were dissolved in Trowell's T8 medium containing $10^{-3}$M sodium ascorbate and 5% 'polypeptide' (Sigma 5115). The steriods were first dissolved in ethanol and then diluted as were the peptide hormones. The final concentration of ethanol was never more than 0.01% which did not influence the ovarian response to LH.

## Results

LH altered the reducing potential of the corpus luteum in sections of ovarian tissue. The response depended on the length of time for which the sections were incubated with the hormone. The maximal change in reducing potential occurred after 80 s

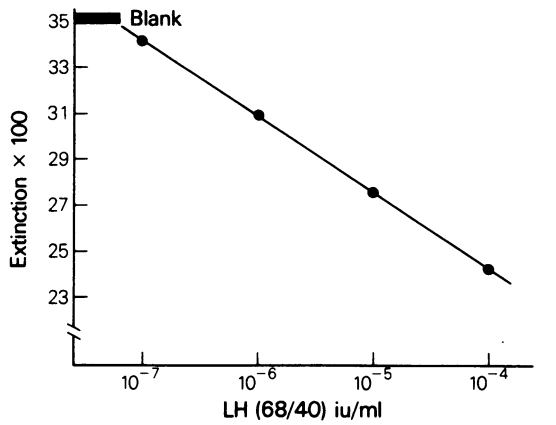


**Figure 2** Relationship between stain intensity and concentration of LH. Every point is the mean of 5 determinations (10 readings from each of 5 sections). Standard errors were within ± 1% of the mean.

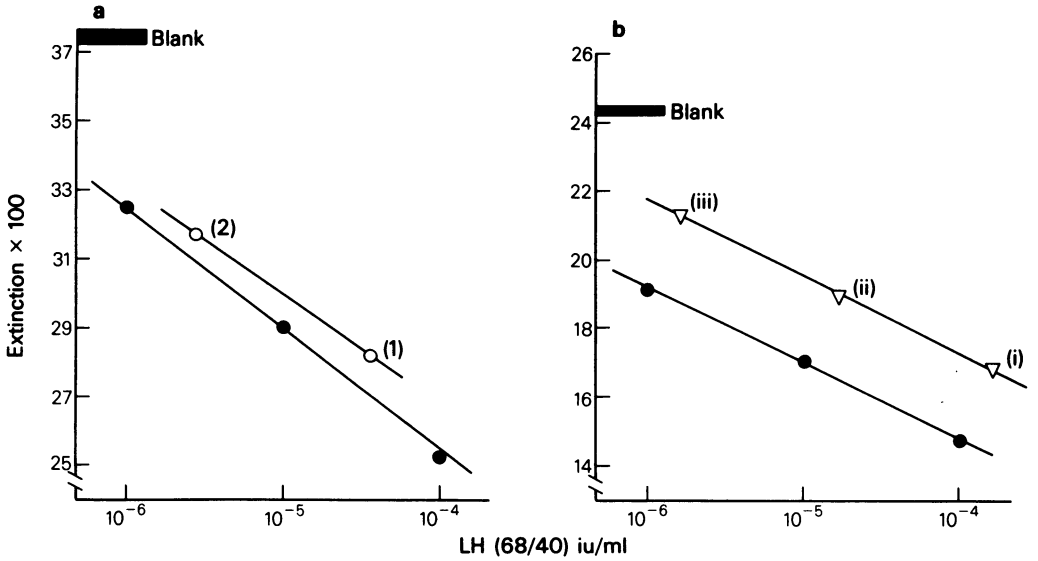


**Figure 3** Comparison of dose-response lines of standard LH with those of (a) plasma and (b) pituitary extracts from rats. (●) LH (68/40); (○) plasma diluted (1) 1 in $10^2$ (2) 1 in $10^3$; (▽) pituitary extract diluted (i) 1 in $10^6$ (ii) 1 in $10^7$ (iii) 1 in $10^8$. Every point is the mean of duplicate determinations.

(Figure 1) which was the incubation time used in all subsequent experiments.

There was an inverse linear relationship between the logarithm of the concentration of LH and the intensity of the stain over a range of concentrations of the hormone from $10^{-7}$ to $10^{-4}$ iu/ml (Figure 2). The dose-response lines obtained using plasma or pituitary extracts from rats (Figure 3) or human plasma (Figure 4a) and those of the International Standard preparation did not deviate significantly from parallelism but with plasma from owl monkeys (Figure 4b) or chickens (Figure 4c) there was always a significant deviation. Acid extracts prepared from the cerebral cortices of rats were completely devoid of activity.

The mean index of precision ($\lambda = s/b$ where s is the standard deviation of the slope and b is the slope) of 10 dose-response lines was $0.12 \pm 0.01$ and the fiducial limits ($P = 0.95$) for a typical 2 + 2 assay of rat plasma LH using 5 sections per point were 88.3 to 113.2%. In a series of 50 assays the fiducial limits were always within ± 15%. Since these limits were so narrow all subsequent data were obtained using only two sections per point. Every assay was done on the '3 + 2' basis, i.e. using three dilutions of standard and

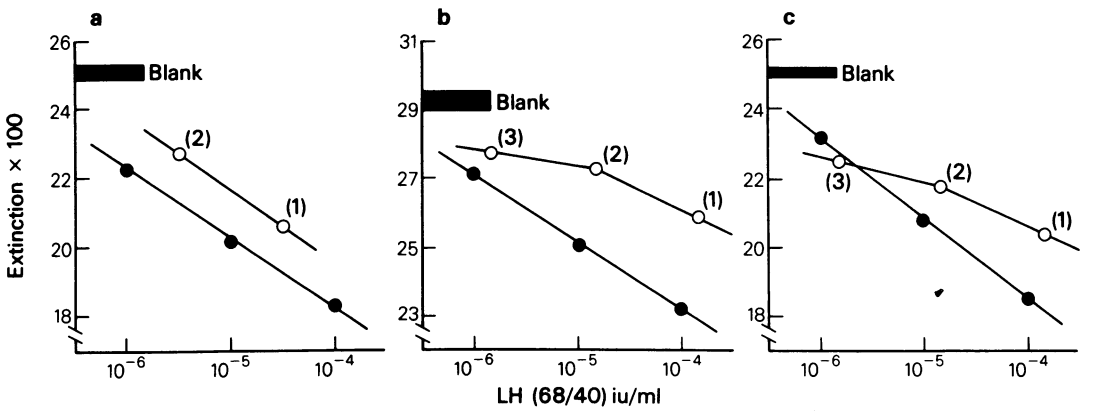


**Figure 4** Comparison of dose-response lines of standard LH with those of plasma from (a) man (b) owl monkey (c) chicken. (●) LH (68/40); (○) plasma diluted (1) 1 in $10^2$ (2) 1 in $10^3$ (3) 1 in $10^4$. Every point is the mean of duplicate determinations.

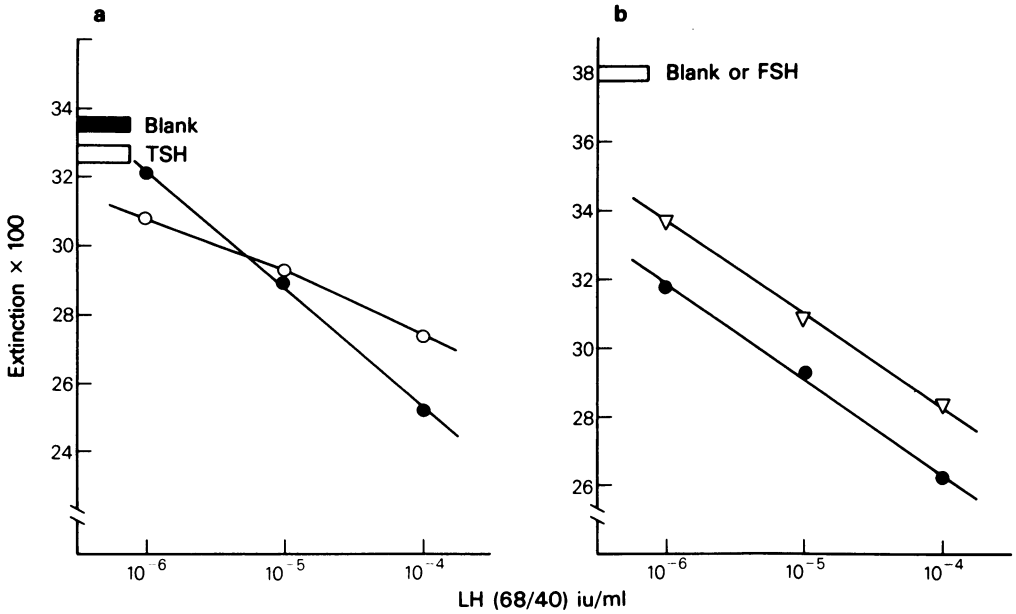


**Figure 5** Effect of (a) TSH (50 μu/ml) and (b) FSH (20 mu/ml) on the ovarian response to LH. (●) LH; (○) LH + TSH; (▽) LH + FSH. Every point is the mean of duplicate determinations.

two of test and in the specificity studies, every assay was repeated four or five times. The method is accurate and the mean recovery of standard LH added to rat plasma was 100% (range 93 to 104% for 6 estimations).

The method appears to be highly specific for LH. Oestradiol (10 ng/ml), testosterone (1 ng/ml), progesterone (1 ng/ml), corticosterone (100 ng/ml), lysine vasopressin (2 mu/ml), angiotensin II (10 ng/ml), prolactin (100 ng/ml), corticotrophin (15 iu/ml), FSH (10 mu/ml) and TSH (5 μu/ml) possessed no intrinsic activity and did not influence the ovarian response to LH. However, in concentrations considerably greater than those ever likely to occur in samples diluted appropriately for assay, TSH (50 μu/ml) and FSH (20 mu/ml) were biologically active. In the higher concentration TSH had some intrinsic activity and also reduced the slope of the dose-response line to LH while FSH appeared to act as a competitive antagonist of LH (Figure 5). Al-

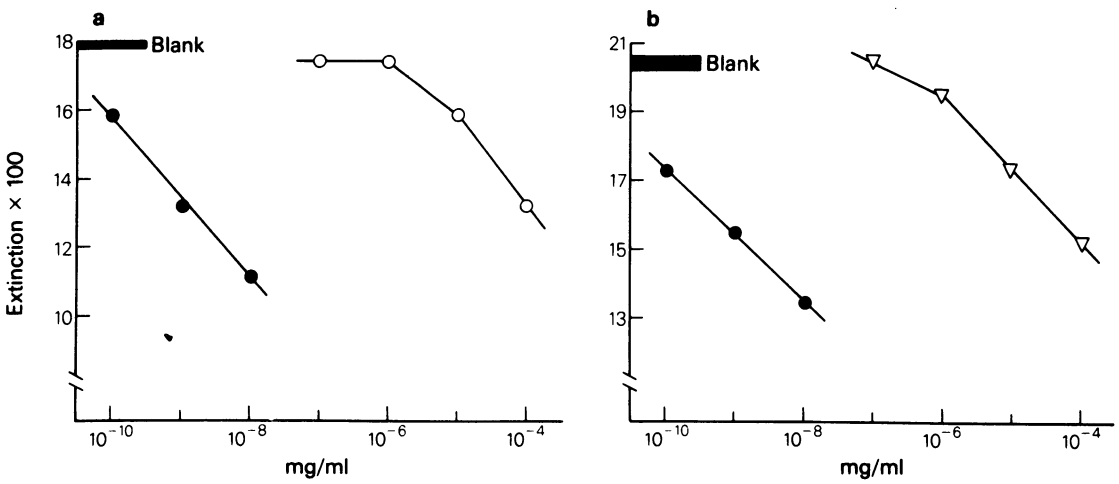


**Figure 6** Comparison of the dose-response lines of standard LH with those of (a) α- and (b) β-subunits of LH. (●) LH (68/40); (○) α-subunit; (▽) β-subunit. Every point is the mean of duplicate determinations.

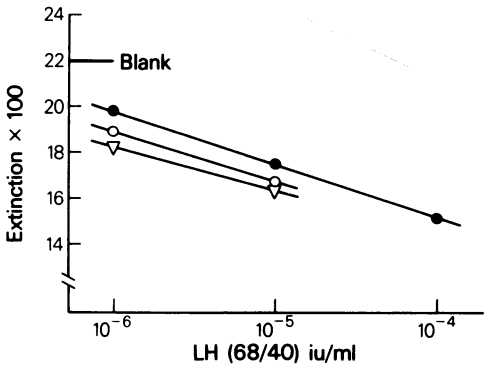


**Figure 7** Effects of α- and β-subunits of LH on the ovarian response to standard LH. (●) LH (68/40); (○) LH + α-subunit (10 ng/ml); (▽) LH + β-subunit (10 ng/ml). Every point is the mean of duplicate determinations.

though on a weight-for-weight basis the α- and β-subunits of LH were 5 to $10 \times 10^4$ times less active, their dose-response lines were parallel with (Figure 6) and their effects additive to (Figure 7) those of the standard preparation of LH. HCG and its subunits were also less active than LH but their dose-response lines were flatter than those of standard LH (Figure 8) with which their effects appeared to be additive (Figure 9).

Blood samples taken throughout the menstrual cycle from 5 normal women, aged 21 to 35 years, exhibited the typical profound increase in LH activity at the mid-cycle. For the sake of clarity the data from only two are shown in Figure 10 but similar LH profiles were observed in the other subjects.

## Discussion

Recently several highly sensitive and very precise biological methods for the determination of LH (Dufau, Mendelson & Catt, 1974; Qazi, Romani & Diczfalusy, 1974) have been developed. Their existence does not diminish the need for alternative techniques since it is only when assays of the same sample against the same standard using several different bioassay systems give identical results that it can be assumed that the test substance is biologically identical with the standard preparation.

The cytochemical section method described in this paper appears to fulfil the essential requirements of a biological assay system for the determination of LH. It is accurate and precise. It is also sensitive enough to detect and measure LH in plasma in concentrations as low as $10^{-5}$ iu/ml. It possesses a high degree of specificity and although TSH, FSH and HCG in high concentrations can influence the ovarian response to LH, it is unlikely that they would ever be present in samples diluted for assay in sufficient quantity to interfere. The parallelism between the dose-response lines obtained using international standard LH (68/40), human and rat plasma and rat pituitary extracts indicates that the method is valid for the determination of the hormone in both these species. The marked deviation from parallelism of the appropriate dose-response lines suggests that chicken and owl monkey LH are different from one another and from the international standard preparation. In this context it is interesting that owl monkey plasma appears to contain no immuno-reactive LH (R. Bonney, personal communication). Like other biological assay procedures this method requires skill. However, with the necessary expertise, one worker can

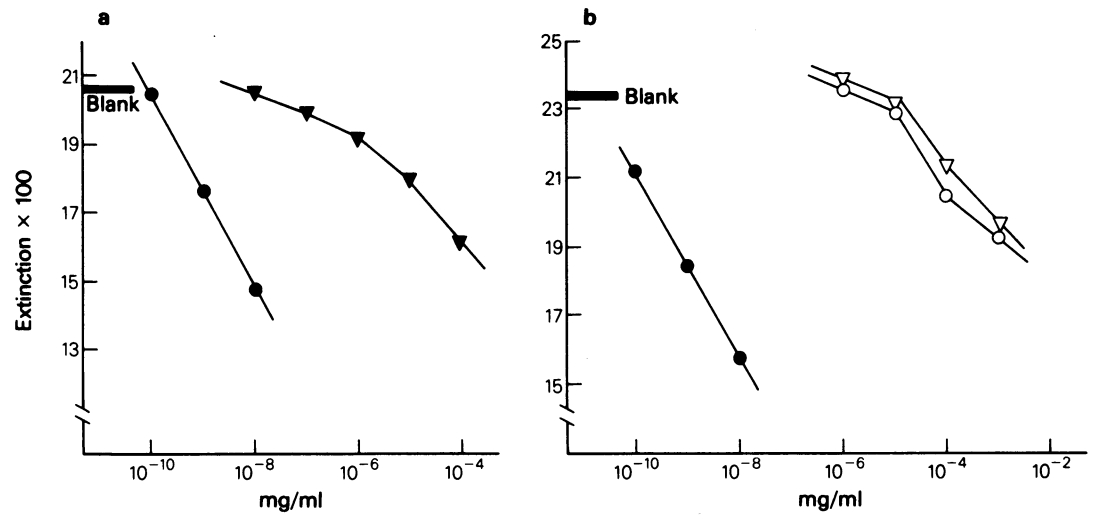


**Figure 8** Comparison of the dose-response lines of standard LH with those of (a) HCG and (b) α- and β-subunits of HCG. (●) LH (68/40); (▼) HCG; (○) α-subunit; (▽) β-subunit. Every point is the mean of duplicate determinations.

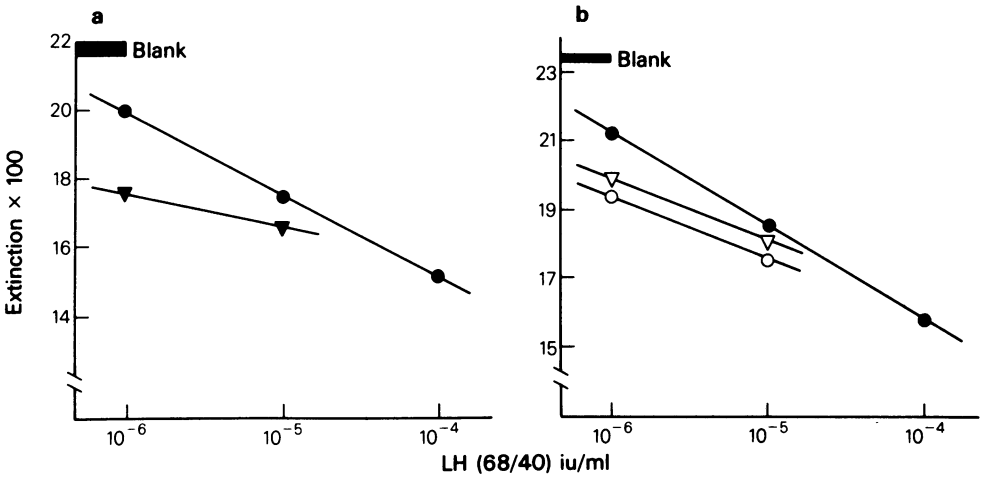


**Figure 9** Effects of (a) HCG and (b) α- and β-subunits of HCG on the ovarian response to standard LH. (●) LH (68/40); (▼) LH + HCG (100 ng/ml); (○) LH + α-subunit (1 μg/ml); (▽) LH + β-subunit (1 μg/ml). Every point is the mean of duplicate determinations.

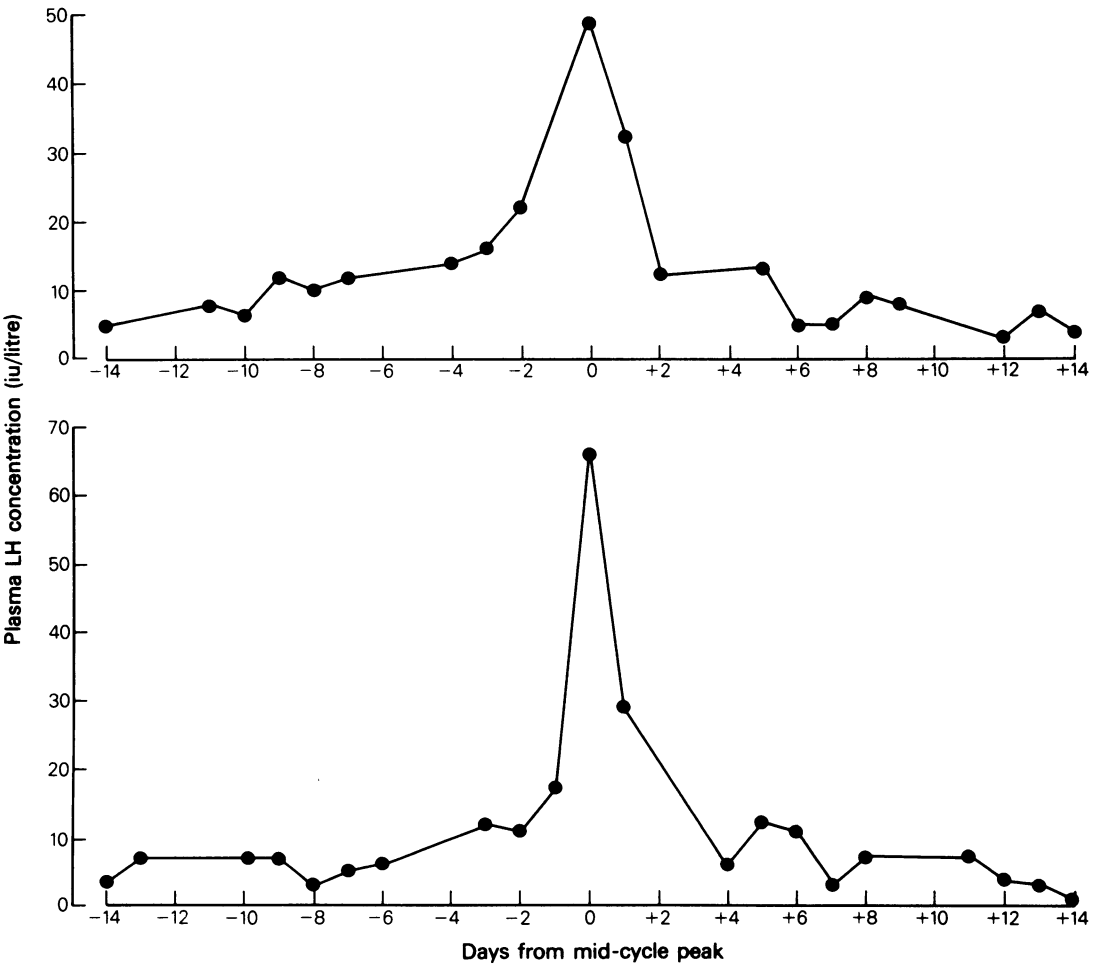


**Figure 10** Plasma concentrations of LH during the menstrual cycle in two healthy young adult women. Every point is the mean of duplicate determinations.

easily process up to 100 samples per week. Furthermore, the initial outlay for equipment and the routine running costs compare favourably with those of other methods.

The changes in the concentration of LH in the plasma during the menstrual cycle were similar to those shown previously using different assay methods. In all of our subjects the maximal concentrations was attained approximately 14 days before the onset of menstruation. Studies using radioimmunoassay methods suggest that the mid-cycle rise in plasma LH lasts for only 2 to 3 days. Our results indicate that it persists for a longer period as was also shown by Watson (1972) and Romani, Robertson & Diczfalusy (1976) using different bioassay methods. Our results are not in agreement with those of Holdaway, Kramer, McNeilly, Rees & Chard (1974) and Kramer, Holdaway, Crighton, McNeilly, Rees & Chard (1976) who claimed that their cytochemical method using segments of 'super-ovulated' ovaries showed the existence of two peaks in the concentration of LH in the plasma during the menstrual cycle. However, the magnitude of their standard errors makes their data difficult to interpret. Although the pattern of changes described here in the concentration of LH in the plasma during the cycle closely resembles that shown previously using radioimmunological methods there are many reports in the literature (e.g. Robertson & Diczfalusy, 1977) of discrepancies between the results obtained with bioassay and radioimmunassay techniques for the determination of LH. It is well known that the latter 'measure antigenic activity that is not necessarily related to biological activity' (WHO technical report, series 565, p.30, 1975). Bioassay methods are essential for the validation of results obtained using radioimmunoassay methods for Gaddum's statement 'when biological methods and chemical methods for the assay of a pharmacologically active substance disagree so widely that the disagreement cannot be due to the error of the test, the biological method is, by definition, right and the chemical method is wrong' (Burgen & Mitchell, 1978) could apply to immunoassays as well as to chemical methods. Cytochemical methods have already resulted in important advances in endocrinology and it is not unreasonable to expect that their exploitation for the determination of LH will extend our knowledge of the hypothalamo-pituitary-gonadal system.

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**References**

ALAGHBAND-ZADEH, J., DALY, J.R., BITENSKY, L. & CHAYEN, J. (1974). The cytochemical section assay for corticotrophin. *Clin. Endocr.*, **3**, 319–327.

ALAGHBAND-ZADEH, J., DALY, J.R., TUNBRIDGE, R.D.G., LOVERIDGE, N. & CHAYEN, J. (1973). Methodological refinements in the redox bioassay for adrenocorticotrophin. *J. Endocr.*, **58**, xix.

BUCKINGHAM, J.C., CHAYEN, J. HODGES, J.R., ROBERTSON, W.R. & WEISS, J. (1979a). A cytochemical section assay for the determination of luteinizing hormone. *J. Endocr.*, **81**, 160P.

BUCKINGHAM, J.C., CHAYEN, J., HODGES, J.R., ROBERTSON, W.R. & WEISS, J. (1979b). A cytochemical section assay method for the determination of luteinizing hormone in human and rat plasma. *Acta endocr.*, Suppl. **225**, 131.

BURGEN, A.S.V. & MITCHELL, J.F. (1978). In *Gaddum's Pharmacology*, p.229. Oxford University Press.

CHAYEN, J., LOVERIDGE, N. & DALY, J.R. (1972). A sensitive bioassay for adrenocorticotrophic hormone in human plasma. *Clin. Endocr.*, **1**, 219–233.

DUFAU, M.L., MENDELSON, C.R. & CATT, K.G. (1974). A highly sensitive in vitro bioassay for luteinizing hormone and chorionic gonadotropin: testosterone production by dispersed Leydig cells. *J. clin. Endocr. Met.*, **39**, 610–613.

HOLDAWAY, I.M., KRAMER, R.M., McNEILLY, A.S., REES, L.H. & CHARD, T. (1974). Applications of the redox bioassay for luteinizing hormone. *Clin. Endocr.*, **3**, 383–388.

KRAMER, R., HOLDAWAY, I.M., CRIGHTON, D.B., McNEILLY, A.S., REES, L.H. & CHARD, T. (1976). Comparison of the redox bioassay with other assays for luteinizing hormone. *J. Endocr.*, **69**, 205–211.

PARLOW, A.F. (1958). A rapid bioassay method for LH and factors stimulating LH secretion. *Fedn. Proc.*, **17**, 402.

QAZI, M.H., ROMANI, P. & DICZFALUSY, E. (1974). Discrepancies in plasma LH activities as measured by radioimmunoassay and an in vitro bioassay. *Acta. endocr.*, **77**, 672–685.

REES, L.H., HOLDAWAY, I.M., KRAMER, R., McNEILLY, A.S. & CHARD, T. (1973). New bioassay for luteinizing hormone. *Nature*, **244**, 232–234.

ROBERTSON, D.M. & DICZFALUSY, E. (1977). Biological and immunological characterisation of human luteinizing hormone: II. A comparison of the immunological and biological activities of pituitary extracts after electrofocusing using different standard preparations. *Mol. cell. Endocr.*, **9**, 57–67.

ROMANI, P., ROBERTSON, D.M. & DICZFALUSY, E. (1976). Biologically active luteinizing hormone (LH) in plasma. I. Validation of the in vitro bioassay when

applied to plasma of women. *Acta endocr.*, **83,** 454–465.

SAYERS, M.A., SAYERS, G. & WOODBURY, L.A. (1948). The assay of adrenocorticotrophic hormone by the adrenal ascorbic acid depletion method. *Endocrinology*, **42,** 379–384.

STORRING, P.L., BANGHAM, D.R., COTES, P.M., GAINES, P.E. & JEFFCOATE, S.L. (1978). The International Reference Preparation of human pituitary luteinizing hormone for immunoassay. *Acta endocr.*, **88,** 250–259.

WATSON, J. (1972). Plasma levels of luteinizing hormone in women and in rats as measured by bioassay. *J. Endocr.*, **54,** 119–123.

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