OVARIAN STEROIDOGENESIS IN NORMAL AND INFERTILE WOMEN

A Thesis
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of
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by

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ABBREVIATIONS

The abbreviations, symbols and conventions recommended by the IUPAC-IUB Commission on Biochemical Nomenclature and published in Biochem. J. (1973) 131, 1-20, have been used throughout this thesis.

In addition the following abbreviations have been used :-

CPB	Competitive protein binding.
RIA	Radioimmunoassay.
HCG	Human chorionic gonadotrophin.
LH	Luteinizing hormone.
FSH	Follicle stimulating hormone.
'Pergonal'	Human menopausal gonadotrophin (supplied by Searle Scientific Services).
'Clomid'	Clomiphene citrate (supplied by Merrell-National Laboratories Ltd., London W.1.).
SPS	Standard protein solution.
CBG	Corticosteroid binding globulin.
BSA	Bovine serum albumin.
S.D.	Standard deviation.
BBT	Basal body temperature.
I.U.D.	Intra-uterine Device.

STEROID NOMENCLATURE

The IUPAC-IUB 1967 Revised tentative rules for steroid nomenclature have been used in this thesis. These appear in Biochem. J. (1969) 113, 5.

The trivial names set out in J. Endocrinol. (1972), 54, 1-2, have been used without definition. These include :-

corticosterone

cortisol

dehydroepiandrosterone (DHA)

deoxycorticosterone (DOC)

oestrone

oestradiol

oestradiol-17α

oestriol

progesterone

testosterone

17α-hydroxyprogesterone

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CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Historical Background

Basic knowledge of the ovary as an organ of internal secretion stems from the demonstration of involutionary changes in the uterus and vagina following bilateral oophorectomy and their reversal by administration of active ovarian extracts (Marshall and Jolly, 1905). The oestrus-inducing properties of ovarian tissue were first demonstrated by Allen and Doisy (1923) who used the test of vaginal cornification in spayed rodents (Allen, 1922) as a specific index of oestrogenic activity. Chemical identification of the active principle in this material followed and the first preparation of oestrogens in crystalline form was accomplished simultaneously by workers in several countries (see reviews by Allen et al., 1939 and Marrian, 1950).

The physiological significance of the corpus luteum was first realised by Fraenkel (1903; 1910) who demonstrated that this structure had an endocrine function and produced a secretion which was important for the protection of the fertilized ovum. Progesterone was first extracted from luteal tissue in sows' ovaries by Corner and Allen (1929) and shortly afterwards isolated and chemically identified.

A functional interrelationship between the ovary and the pituitary gland was recognised early in this century (e.g. Crowe

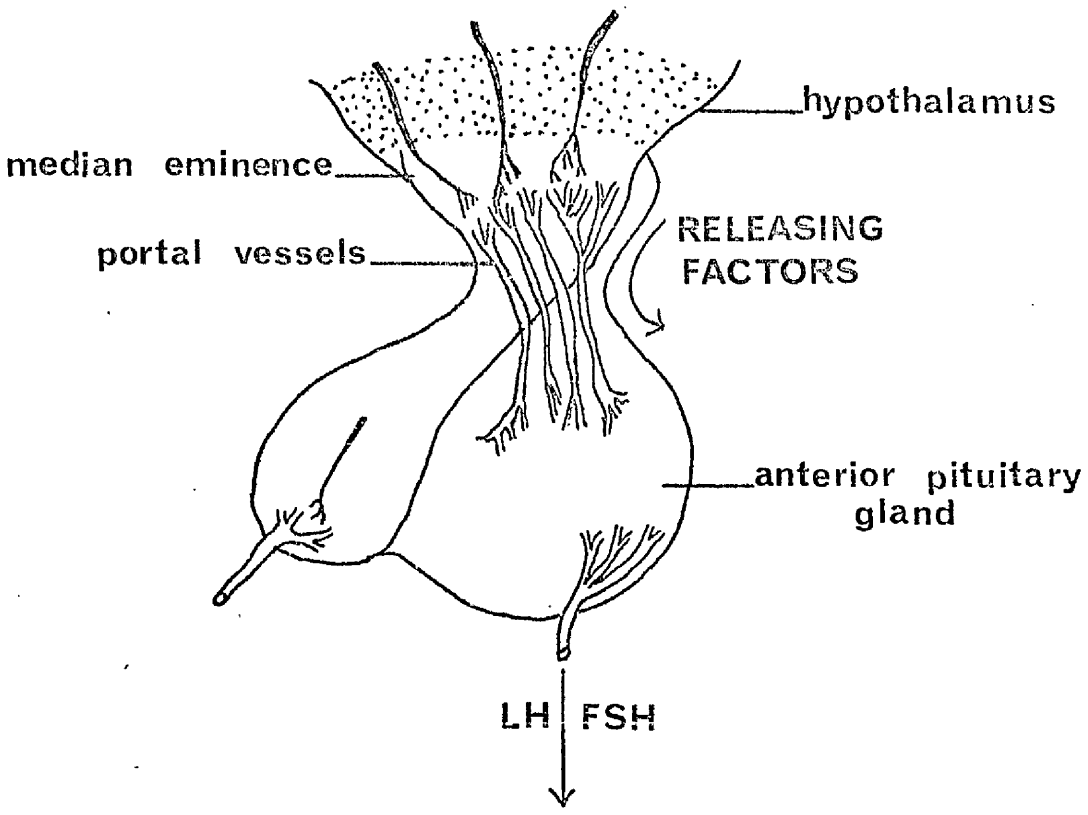
et al., 1910) and readily confirmed with the technique of hypophysectomy (see Smith, 1939). Ovarian changes are continuous and normally cyclical in nature and can be divided into two phases characterised by follicular development and formation of a corpus luteum. Following the observation by Smith (1927) of a follicular response to pituitary implants and the finding of Evans and Simpson (1928) that alkaline pituitary extracts induced luteinization, the existence of two separate gonadotrophic factors was postulated (Wiesner and Crew, 1930). At the same time, two fractions from the anterior lobe of the pituitary were prepared, one with follicle stimulating and the other with luteinizing properties (Fevold et al., 1931). Thus the two factors, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were discovered. It now became clear that the ovary formed part of the female reproductive system as a whole. As such its functions could not be considered in isolation but in the context of a chain of organs extending from the pituitary to the external genitalia and linked by an intricate and closely integrated series of endocrine processes.

The major unexplained phenomenon was now that of the mechanism of the interaction of the ovary and the pituitary in producing a series of cyclical events which were repeated at regular intervals. It had been realised that some connection

existed between ovarian function and the nervous system. Many factors suggested that the cycle of events in the mammal lay primarily under nervous control. In lower mammals for example, the sex cycle of the ferret depended upon the length of daylight to which the animal was exposed whilst in women, amenorrhoea was known to develop under psychological stress. The concept of nervous control of the anterior pituitary was postulated by Marshall (1937). The first evidence which showed this hypothesis to be feasible came from Green and Harris (1947) with the demonstration of the hypothalamo-hypophyseal portal circulation by which a connection was established between the hypothalamic nerve endings and the adenohypophysis. However, it was not until the 1960 s (Harris, 1961; Nikitovitch-Winer, 1962) that direct evidence was provided in support of this concept. It is now well documented that gonadotrophin releasing factors are produced at hypothalamic nerve endings lying near the primary capillary plexus of the hypothalamo-hypophyseal portal system which carries them to the adenohypophysis causing gonadotrophin release. This is illustrated in Fig. 1. I.

It was now clear that all aspects of the ovarian cycle are controlled directly and the endocrine functions of the ovary indirectly by the FSH and LH of the anterior pituitary. The ovary in turn, by means of its own steroid hormone secretions

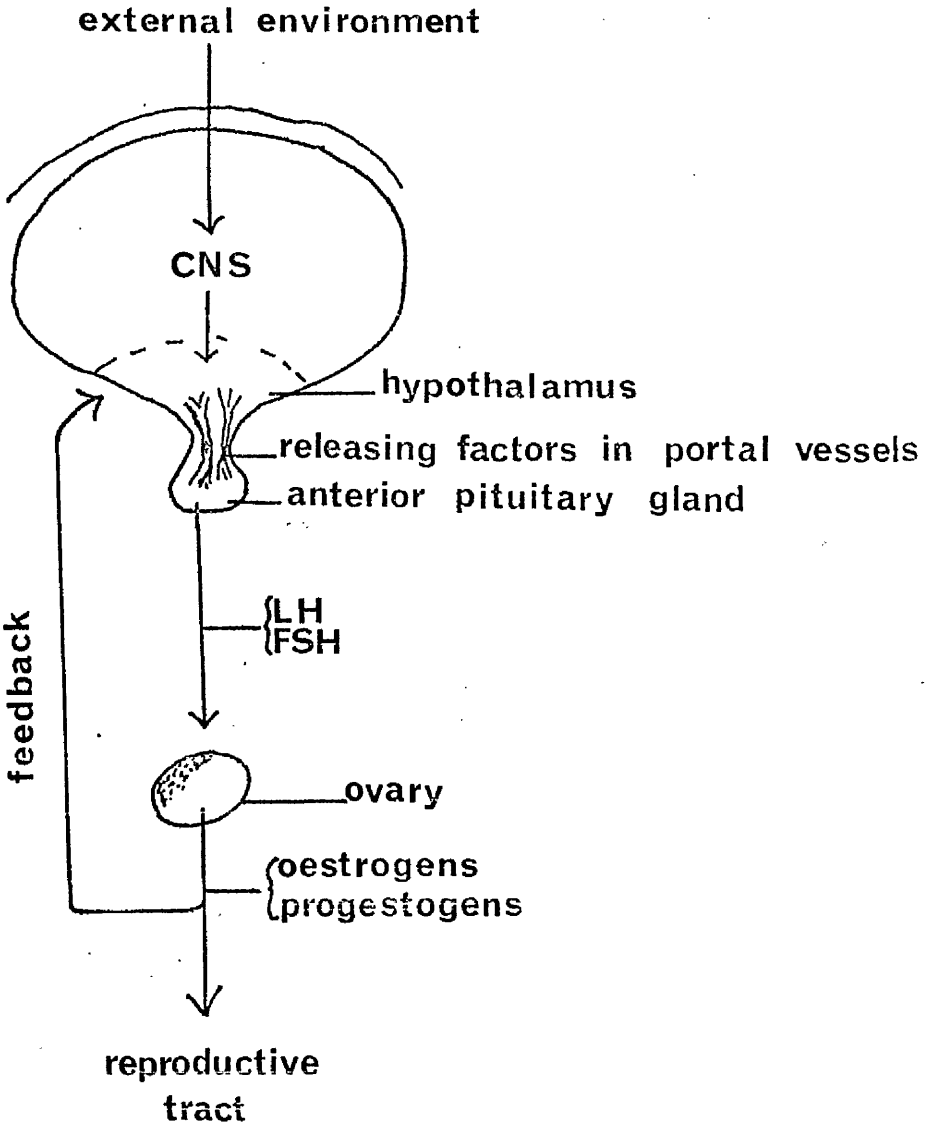
Fig. 1. I SECTION THROUGH THE BASE OF THE HYPOTHALAMUS AND PITUITARY GLAND TO SHOW THE MECHANISMS CONCERNED IN THE REGULATION OF ANTERIOR PITUITARY ACTIVITY



regulates the activity of the accessory sex organs and thus ultimately the sexual cycle. Furthermore, it was postulated that the gonadotrophic activity of the pituitary is itself related by a feed-back mechanism to the concentration of ovarian hormones in the blood. An outline of these interactions is illustrated in Fig. 1. II. To prove these hypotheses, and to investigate the nature of this feed-back mechanism in humans, measurements of the hormones produced by the ovary and the pituitary throughout the menstrual cycle were necessary.

The first quantitative assessments of ovarian function in the menstrual cycle were made by estimating oestrogens and progestogens in the urine. Much of the work on the estimation of oestrogen in urinary extracts prior to 1955 was performed by bioassay techniques which, although sensitive, were relatively imprecise. By means of such procedures a considerable amount of clinical information was accumulated on oestrogen excretion in health (e.g. Smith et al., 1938) and disease (e.g. Pedersen-Bjergaard and Tønnesen, 1951). However, the results so obtained were often of doubtful quantitative significance. The introduction of chemical methods for measurement of oestrogens gave rise to more precise results. These included techniques involving the Kober reaction, originally described by Kober in 1931, (e.g. Brown, 1955a), fluorimetry (e.g. Preedy and Aitken,

FIG. 1. II THE CENTRAL NERVOUS - PITUITARY - OVARIAN AXIS



1961) and gas chromatography (e.g. Menini, 1965). Studies by Brown (1955b) and Brown et al., (1958) showed that there was a peak in the urinary oestrogen excretion pattern at about the middle of the human menstrual cycle followed by a rapid decrease in oestrogen excretion, which was followed by a second oestrogen rise during the luteal phase of the cycle.

The first quantitative study of progestogen excretion was by Venning and Browne (1937) who measured urinary pregnanediol, which was later shown to be exclusively derived from progesterone (see Eckstein, 1962) throughout the cycle. The technique of Venning (1937) was a gravimetric one and more recently pregnanediol has been measured by two main methods, colourimetry (e.g. Klopper et al., 1955) and gas chromatography (e.g. Goldzieher et al., 1967). Klopper (1957) showed that low levels of pregnanediol were excreted in the follicular phase of the cycle, with levels rising from mid-cycle to a peak in the luteal phase. It was realised however, that urinary pregnanediol determinations are not truly representative of endogenous progesterone production but only a reflection of the output of the hormone (Klopper and Billewicz, 1963). Macnaughton and Greig (1965) have shown that only between 6% and 15% of $\left[{}^{14}C\right]$ progesterone injected into pregnant or non-pregnant women appears as pregnanediol in the urine. Thus methods involving the measurement of all hormones directly in the blood were sought.

Measurements of oestrogens in blood were originally carried out using the same type of technique as for urine; methods involved the Kober reaction (e.g. Roy and Brown, 1960), gas chromatography (e.g. Wotiz et al., 1967) and double isotope derivative formation (Baird, 1968). Progesterone in blood was measured by spectrophotometric methods (e.g. Short, 1958), gas chromatography (e.g. Kumar et al., 1964) and isotopic methods (e.g. Woolever and Goldfien, 1963). Estimations in peripheral blood provided essentially the same information as urinary estimations. However, although these methods were specific they were relatively insensitive and large amounts of blood were required for assays. Thus samples were often pooled either within subjects or over several days in order to obtain sufficient blood. At the same time it was becoming obvious that the human menstrual cycle was characterised by marked fluctuations in endogenous hormone levels, so that little detailed information could be obtained from pooled or random samples. An accurate assessment of ovarian function in the reproductive cycle could only be obtained by serial sampling in individual subjects and to make this study a practical undertaking more sensitive methods of assay were required.

The study of pituitary function in the human menstrual cycle faced similar problems. The first measurements of pituitary gonadotrophins involved determination of 'total

gonadotrophin activity' by bioassays such as the mouse uterus test of Klinefelter et al., (1943). Procedures were soon introduced for the separate determination of FSH and LH involving bioassays carried out in urine. FSH was measured using the ovarian augmentation test (Steelman and Pohley, 1953) and LH by the ovarian ascorbic acid depletion test (Parlow, 1961). Using these tests, a peak of LH excretion was reported to occur always at mid-cycle and this peak was closely associated with ovulation as judged by urinary steroid assays (Brown et al., 1958; McArthur et al., 1958). Bell et al., (1966) studying FSH output showed great variations in the pattern of FSH excretion from one woman to another. In some subjects levels did not alter throughout the cycle; in others levels were maximal at the time of ovulation, whilst in others the highest levels occurred in the follicular phase. Conversely, Fukushima et al., (1964) claimed that the curve of FSH output during the cycle tended to be constant with high levels in the follicular and luteal phases and low levels at mid-cycle.

Perhaps the greatest single breakthrough in the study of reproductive endocrinology was the introduction of the technique of displacement analysis. This technique was first used by Berson and Yalow (1957) to assay insulin and later applied to the measurement of gonadotrophins (e.g. Bagshawe et al., 1966)

and steroid hormones (Murphy, 1964). The principle of this method will be described in detail in Chapter 3. Briefly, it depends on the displacement of a substance, X, labelled with a radioactive isotope, from a binding protein, by unlabelled X. Subsequent measurement of the bound and/or free isotopically labelled X allows estimation of the mass of unlabelled X present by comparison with standards. Such methods provide a high degree of sensitivity making them applicable to assay of hormones in small volumes of biological fluids. It is therefore now possible to assay steroid and gonadotrophin hormones in daily blood samples from individual subjects throughout entire menstrual cycles.

Background and Scope of the Present Study

Recent studies of ovarian and pituitary function and their interaction have involved the correlation of morphological changes with hormone secretion patterns in daily samples throughout the menstrual cycle. Three steroid hormones, progesterone, 17α-hydroxyprogesterone and oestradiol, have frequently been studied, together with the gonadotrophins, LH and FSH. Each of the steroid hormones has been used to monitor certain aspects of ovarian function.

In vitro studies indicate that the major source of ovarian progesterone production is the granulosa cells (Ryan and Petro, 1966) which become luteinized and form the corpus luteum. The increased levels of progesterone found in the luteal phase of the menstrual cycle have thus been regarded as an index of ovulation and of corpus luteum function. There has been speculation as to the absolute level of progesterone which signifies an ovulatory cycle (Isreal et al., 1972; Black et al., 1972). However, Newton (1973) has suggested that the area under the progesterone curve in the luteal phase of the cycle is perhaps a more accurate measure of corpus luteum activity and function. Increasing plasma concentrations of progesterone are reflected in increasing basal body temperatures (Davis and Fugo, 1948; Isreal and Schneller, 1950) and a biphasic basal body temperature curve has been regarded as a useful indicator of ovulation (Hartman, 1962).

The processes of corpus luteum function and follicular maturation however, must be examined separately as whilst development of a corpus luteum is usually linked with ovulation, the two processes may be dissociated. Such dissociation would be impossible to detect if evidence of ovulation was based solely on corpus luteum function through increased progesterone secretion. Strott et al., (1969) have measured 17α-hydroxyprogesterone in human plasma and showed that a peak occurs in the secretion

pattern on the day prior to, or the day of the LH mid-cycle peak and they suggested that this pre-ovulatory rise could be regarded as a measure of follicular maturation. The pattern of variation of 17α-hydroxyprogesterone is similar to that observed by Fotherby (1962) for urinary pregnanetriol, thought to be derived from 17α-hydroxyprogesterone.

Patterns of oestrogen secretion have been used to monitor follicular growth during the pre-ovulatory phase of the menstrual cycle. Oestradiol-17β is the major oestrogenic product of the growing follicle, as shown by studies on ovarian vein blood (Mikhail, 1967). In vitro studies indicate that the theca cells of the developing follicle are the major site of synthesis of oestradiol (Ryan et al., 1968). The pattern of oestradiol in the luteal phase of the cycle closely resembles that of progesterone, which suggests that this oestradiol may be produced by the corpus luteum, or at least by the theca cells which persist within the corpus luteum (Fawcett, 1968). If this were so, oestradiol may be useful as a marker of both follicular growth and corpus luteum function.

The relationship of these steroid hormones, each marking some aspect of ovarian function, to the gonadotrophins may indicate their role in mediating the pituitary response to morphological changes occurring during the cycle. Several

recent reviews describe the present state of knowledge concerning ovarian pituitary interactions, gained by studies of hormone patterns in the menstrual cycle (Henzle and Segre, 1970; Ross et al., 1970; Vande Wiele et al., 1970). They indicate that whilst there is now much information available about individual events, there is still much controversy over certain aspects of this interaction.

The most dramatic single event in the menstrual cycle is the mid-cycle surge of LH, which precedes ovulation of a mature follicle. The pattern of FSH secretion is not so marked; higher levels are found in the follicular phase than in the luteal phase. The follicular phase release of FSH appears to be required for stimulation of growing follicles resulting in an increased output of oestradiol. A peak of FSH appears at mid-cycle, usually coincident with the LH peak. No role has yet been assigned to this mid-cycle peak. The ovarian steroids exert a negative feed-back effect on LH and FSH. There is a strong inhibition of FSH secretion in 'fertile life' - only 5 - 10% of the levels found in oophorectomised or post-menopausal women appear during the menstrual cycle. This feed-back effect is smaller on LH, where only 30 - 40% inhibition, calculated from post-menopausal levels, occurs in the 'fertile' period. However, at mid-cycle, for both gonado-

trophins a transient positive feed-back mechanism seems to function. It is possible that this changeover occurs through fluctuations in the levels of one, or more, steroid hormones.

Both oestrogens and progesterone have been implicated as triggers of the ovulatory surge of LH. Progesterone has been shown to induce or to advance ovulation in rats (Nallar et al., 1966). Odell and Swerdloff (1968) studying plasma FSH and LH levels in castrates and menopausal women, whose elevated gonadotrophin levels had been depressed by the administration of oestrogens, showed that progesterone (10 mg given intramuscularly) will produce a sudden rise in LH and FSH. This, in conjunction with the fact that urinary pregnanediol excretion has been shown to rise on the day before the LH peak (Goebelsmann et al., 1969) makes progesterone a possible trigger of LH release. However, a rise in plasma progesterone prior to a rise in LH has not been demonstrated in women under normal physiological conditions.

There is also evidence which points to oestradiol as a trigger of LH release. Vorys et al., (1965) and Kaiser et al., (1966) report an increase in urinary gonadotrophin following administration of oestrogens. Using a radioimmunoassay to measure LH and FSH in blood, Swerdloff and Odell (1969) present

evidence that synthetic oestrogens will produce repeated bursts of LH but not of FSH. Baird and Guevara (1969) found that plasma oestradiol levels rose continuously from at least the eighth day before the LH peak, and that the peak concentrations of LH and oestradiol were synchronous. However, other workers (Dufau et al., 1970) have found that in some cycles the peak of oestradiol falls before the LH peak so that oestradiol levels are decreasing as LH levels are increasing.

This evidence poses several questions; is progesterone or oestradiol the trigger of LH release ; is LH release triggered separately from FSH, although coincidentally ; could a falling level of oestradiol trigger LH release? Furthermore, other steroids may be involved in this mid-cycle gonadotrophin surge. No role has yet been assigned to the mid-cycle peak of 17α-hydroxyprogesterone described by Strott et al., (1969). After reaching a peak on the day of, or the day preceding the LH peak, levels of this steroid decrease by about 50% and then increase in the luteal phase of the cycle. Progesterone and 17α-hydroxyprogesterone therefore behave independently and Strott et al., (1969) suggest that 17α-hydroxyprogesterone is secreted independently of progesterone and probably by a different cell type. The fluctuations in 17α-hydroxyprogesterone parallel changes in oestradiol and Strott et al., (1969) suggest that

the 17α-hydroxyprogesterone may be secreted as an intermediate in oestrogen biosynthesis. However, this seems unlikely as the levels of 17α-hydroxyprogesterone in peripheral plasma are reported to be of the order of ten times those of oestradiol. It seems unlikely that a precursor should be produced and secreted in such large amounts unless it has some physiological role. Alternatively, 17α-hydroxyprogesterone could be produced independently of oestradiol. In any case, this rise could represent physiologically important pre-ovulatory increases in progestogens.

Detailed investigation of this mid-cycle period could be made in experimental animals by frequent blood sampling or by artificially producing pituitary response to various experimental conditions. It has become increasingly obvious in recent years that experiments in animals may not be applicable to the situation in the human. However, methods of studying the human are obviously limited. There are three main methods which have been used to study menstrual cycle regulation in women; firstly by giving synthetic oestrogens or progestogens and monitoring pituitary response, secondly by following the chronological sequence of events in normal menstrual cycles and thirdly by studying cycles in infertile women, to detect the essential

features which may be absent and how they can be corrected using stimulatory drugs. The first method is of limited use because although the ability of an organ to respond to a stimulus can be demonstrated, it cannot be proved that this event occurs under physiological conditions. The second and third methods of study give a more accurate picture of events as they occur in the 'intact' cycle.

Infertility caused by absence of ovulation has most commonly been studied in this context as it is easily diagnosed either by absence of menstruation or of a mid-cycle LH peak. The differences between anovulatory cycles and normal cycles are likely to be very marked; stimulation of ovulation and subsequent pregnancy may show what features must be induced to produce the fertile cycle. However, an equally useful approach may be made by studying cycles in women who are infertile for no obvious reason, where normal menstruation occurs at regular intervals, and where all other aspects of reproductive function appear clinically normal, but pregnancy does not occur despite a high sperm count in the husband. Such cycles may have more subtle differences in comparison to normal cycles from which useful information may be obtained. This type of infertility also

presents a clinical problem and there are few reports of its successful treatment. Such a study may help to diagnose the condition. If it is assumed that this infertility is caused by some defect in ovarian or pituitary function or their interaction, treatment of these patients with a pituitary stimulatory drug may help to improve their cycles. Clomiphene citrate has been used by many workers as a pituitary stimulatory drug, although usually in the treatment of anovulation (e.g. Macgregor et al., 1968). However, its mode of action is not clear. There is evidence that clomiphene citrate acts through the hypothalamic-pituitary axis to increase gonadotrophin secretion (Jacobson et al., 1968) while other studies indicate that it acts by stimulating the ovary to produce oestrogen, which may trigger LH release (Smith, 1966).

The present work therefore comprises a study of several ovarian hormones, progesterone, 17α-hydroxyprogesterone, oestradiol and gonadotrophins, LH and FSH, in serial daily plasma samples throughout menstrual cycles from a number of normally menstruating women. Comparison of these hormone patterns to patterns in cycles from patients complaining of the type of infertility outlined above before and after treatment with clomiphene citrate may help to elucidate the problem of ovarian-pituitary interaction.

In order to undertake such a study, sensitive and precise methods are required for measurement of the hormones in small volumes of plasma. A competitive protein binding method for the estimation of progesterone has previously been developed in this laboratory (Martin et al., 1970). A useful adaptation of this method would be to incorporate the measurement of 17α-hydroxyprogesterone in the same sample of plasma. An extremely sensitive assay is required for the measurement of oestradiol as this hormone is reported to be present in sub-picomole quantities per milliliter of plasma. A radioimmunoassay technique would be suitable in this context. Facilities are not available in this laboratory for assay of gonadotrophins so these hormones have been assayed elsewhere.

While this work has been in progress several reports of measurements of these hormones in serial samples from normal menstrual cycles have appeared (Johansson et al., 1971; Mishell et al., 1971; Thorneycroft et al., 1971; Abraham et al., 1972; Holmdahl and Johansson, 1972) using different methods from the present work. However, in only one of these studies (Abraham et al., 1972) were all the hormones measured simultaneously. From these reports, several concepts of ovarian-pituitary regulation have been suggested and these

will be discussed in relation to the present study in Chapter 7.

The principle aims of the study described in this thesis were as follows:

1. To develop and evaluate precise and specific methods for the measurement of progesterone, 17α-hydroxyprogesterone and oestradiol in human plasma.

2. To examine ovarian-pituitary interaction by studying the chronological sequence of events occurring in ovarian and pituitary hormone patterns throughout menstrual cycles in a number of normal volunteers.

3. To examine the chronological sequence of events in cycles from selected infertile patients, who apparently menstruate normally, and, after investigation, are found to have patent fallopian tubes and normal secretory endometrium, in order to detect subtle differences which may exist in hormone patterns or levels between such cycles and the normal cycle.

4. To examine the effect which the administration of clomiphene citrate may have on these infertile cycles and to compare

such treatment cycles with the normal cycle.

5. To thus examine the mode of action of clomiphene citrate in such patients and to assess its usefulness in the treatment of this type of infertility.

6. To assess the use of measurement of the steroid hormones studied as markers of the morphological changes occurring in the normal menstrual cycle and in the infertile patient.

CHAPTER 2

MATERIALS AND GENERAL METHODS

MATERIALS AND GENERAL METHODS

Solvents

All solvents used were 'Analar' grade except industrial acetone (BDH Chemicals Ltd.) which was used without purification. The solvents were distilled using a 60 cm Vigreux column (Quickfit, Ltd.). The first and last 100 ml fractions in all distillations were discarded.

Ethyl acetate (BDH Chemicals Ltd.) and methanol (Burrough Ltd.) were distilled twice.

Diethyl ether (BDH Chemicals Ltd.) was distilled twice immediately before use and bottles were discarded for use in radioimmunoassay after half of their contents had been removed.

Light petroleum, boiling range 60° - 80° C (BDH Chemicals Ltd.) was washed with concentrated sulphuric acid and distilled twice.

Pyridine (BDH Chemicals Ltd.) and acetic anhydride (BDH Chemicals Ltd.) were distilled once.

Water was passed through a de-ionizing column (Permutit Co. Mark II portable de-ionizer) and distilled immediately before use in a glass still (Aquator 60E, Anderman and Co. Ltd., London).

Chemicals

Ethylenediaminetetra-acetic acid, disodium salt (EDTA), gelatine powder, di-sodium hydrogen orthophosphate (anhydrous) and sodium dihydrogen orthophosphate ($2H_2O$) were obtained from BDH Chemicals Ltd. 'Analar' grade chemicals were used where available. Thimerosal (Grade II, sodium salt) and 'Trizma base' (reagent grade) were purchased from Sigma Chemical Co. Ltd.

Dextran T40 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Norit NK charcoal (Hopkin and Williams Ltd.) was washed twice with methanol, the 'fines' being decanted each time, and dried thoroughly before use.

Radioactive chemicals

$\left[1,2\text{-}^3H\right]$ progesterone (33.5 Ci/mmol), $\left[1,2\text{-}^3H\right]$ 17α-hydroxyprogesterone (49.2 Ci/mmol), $\left[1,2\text{-}^3H\right]$ testosterone

(45.0 Ci/mmol) and [1,2-^{3}H] corticosterone (44.0 Ci/mmol) were purchased from New England Nuclear Corporation (Frankfurt, Germany). [6,7-^{3}H] oestradiol-17β (40 Ci/mmol) and [1-^{3}H] N-hexadecane (2.27 μCi/g) were purchased from the Radiochemical Centre (Amersham). All radioactive steroids were stored in solution in methanol (100 μCi/ml), at 4°C. The purity of these solutions was checked by paper chromatography before use. No impurities were detected.

Steroids

Progesterone, 17α-hydroxyprogesterone, testosterone, (Steraloids Ltd.), oestradiol, oestriol and oestrone (Koch-Light Laboratories Ltd.) were purchased commercially, purified by paper chromatography and stored in solution in ethyl acetate or methanol at 4°C.

Chromatography

Whatman No.1 chromatography paper from 27 cm wide rolls was cut into the required lengths, washed with methanol in a 2 litre Soxhlet extractor (Quickfit Ltd.) for 48 h, dried in air and stored wrapped in aluminium foil in a dessicator. Before use the lengths were cut into lanes as shown in Fig. 2. I. and rewashed by descending chromatography in methanol.

FIG. 2. I PREPARATION OF CHROMATOGRAPHY PAPER

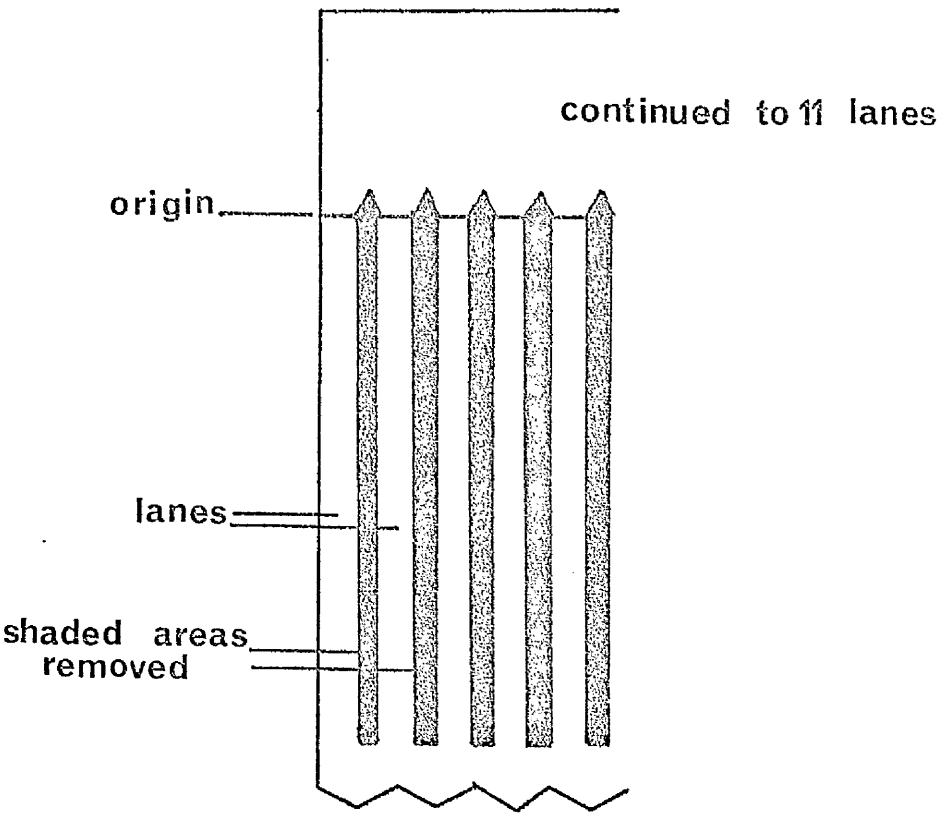
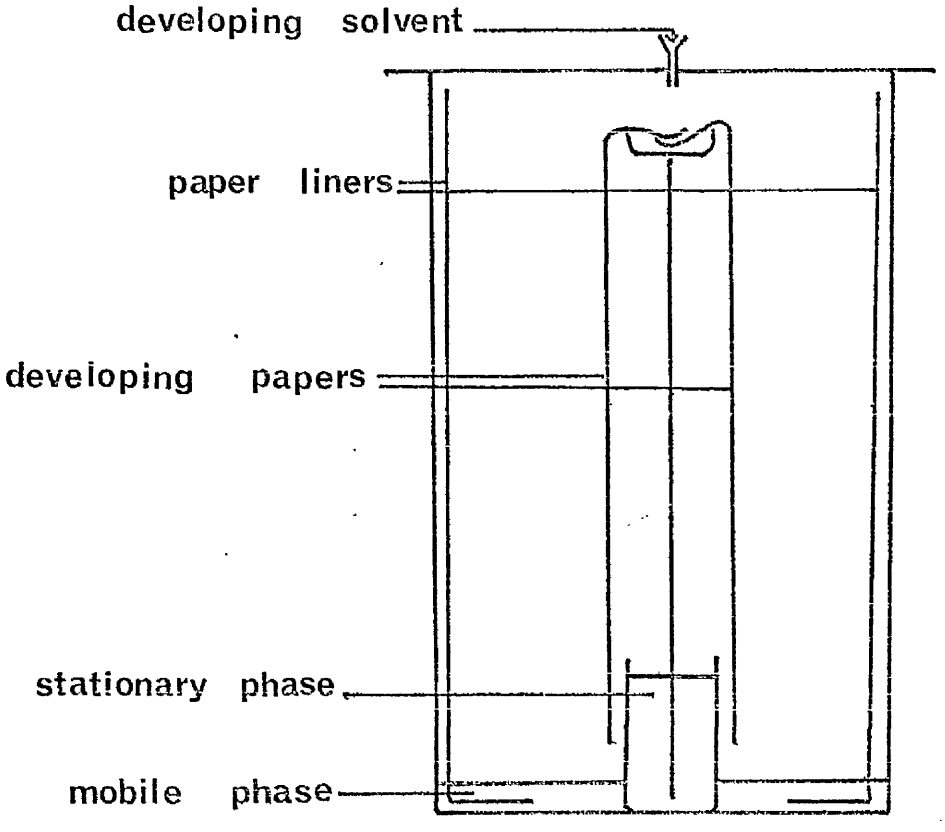


FIG. 2. II PREPARATION OF CHROMATOGRAPHY TANKS



Chromatography tanks (Aimer Ltd.) each held a maximum of four papers. The tanks were lined with chromatography paper and the lids sealed with strips of zinc oxide tape after preparation for chromatography as shown in Fig. 2. II. Chromatograms were equilibrated overnight and the mobile phase added automatically at 06.00 h by means of a time clock and sample changer (The Central Ignition Co. Ltd., London). The chromatograms were removed from the tanks at 09.30 h and lanes containing radioactive standards were scanned. The areas on the sample lanes corresponding to the standards were cut out and eluted with 3 ml of methanol using the syringe and bent needle technique (Dominguez, 1967).

Determination of radioactivity

$[^3H]$ progesterone and $[^3H]$ 17α-hydroxyprogesterone used as chromatography standards were located using a Packard Model 7200 radiochromatogram scanner.

Non-aqueous samples for scintillation counting were dissolved in 8 ml of NE 233 (Nuclear Enterprises Ltd., Edinburgh) liquid scintillant and aqueous samples in 8 ml of NE 250 (Nuclear Enterprises Ltd., Edinburgh) liquid scintillant. Samples were determined for radioactivity in a Nuclear Chicago 'Isocap

300' liquid scintillation spectrometer in which aqueous samples were counted with an efficiency of 49% for $\left[^{3}H\right]$ and non-aqueous samples with an efficiency of 57% for $\left[^{3}H\right]$. Efficiency was checked regularly using a standard stock solution of $\left[1 - ^{3}H\right]$ N-hexadecane.

Blanks (vial and scintillant only) gave values of below 10 cpm consistently and samples were not corrected for this background. Wheaton glass scintillation vials (low background) were used for all samples.

Glassware

Stoppered B14 tubes (MF 24/1/5, Quickfit Ltd.) were used for extraction of plasma and extracts were transferred to conical tubes (BC 24/C14T, Quickfit Ltd.) prior to chromatography. Disposable glass tubes, 12.5 cm by 1.5 cm (Samco Ltd.) were washed in distilled, de-ionized water before use for competitive protein binding assay and radioimmunoassay. Standard flasks were grade B glassware (E-mil). Blow out graduated pipettes (E-mil) were used for measuring plasma samples (1.0 ml and 0.2 ml) and for removing aliquots for radioactivity determinations (0.2 ml and 0.5 ml).

Used scintillation vials were emptied, rinsed with industrial acetone and then, together with all other radioactive glassware,

soaked overnight in Decon 75 (Medical Pharmaceutical Developments Ltd., Sussex) cleaned in an ultrasonic bath (Mettler Electronics) for 30 min, rinsed ten times with cold tap water, a further ten time with de-ionized, distilled water and dried at 100°C. All tubes used in assays were stored in polythene bags prior to use.

Equipment

A refrigerated centrifuge (MSE, 6L) with a multi-load head, capable of carrying up to 96 tubes was used for centrifugation of charcoal.

'Whirlimixers' (Fisons Scientific Apparatus Ltd.) were used for routine mixing after addition of reagents.

A magnetic stirrer (Toyo, supplied by Chem Lab. Instruments Ltd.) was used to stir suspensions of dextran coated charcoal to ensure homogeneity of sampling.

'Repettes' (10.0 ml and 2.0 ml), supplied by Jencons Ltd., were used for repeated additions of reagents in the competitive protein binding and radioimmunoassay methods. Attachment of a fine gauge needle to the 2.0 ml 'repette' was found convenient for additions of 100 µl volumes in the radioimmunoassay.

A 10 µl repeating Hamilton syringe (V.A. Howe, Ltd.) was used for addition of $\left[{}^{3}H\right]$ internal standards to plasma prior to extraction.

Buffers and Solutions

1. Competitive protein binding assay

0.01M-Tris, 0.001M-EDTA buffer was prepared in distilled, de-ionized water and the pH adjusted as required with 2M-HCl.

Dextran coated charcoal (0.25% charcoal, 0.025% dextran) and 0.5% gelatine solutions were prepared in the above buffer.

Stock solutions of progesterone and 17α-hydroxyprogesterone (10ng/ml) were prepared in ethyl acetate.

Standard Protein Solution (SPS)

The protein was contained in the plasma of a post-menopausal woman after treatment with 1.25 mg of ethinyl oestradiol per day for fourteen days together with 2.0 mg per day of dexamethasone for the final three days. This plasma was stored in 0.5 ml aliquots at -20°C. The binding capacity was not affected by thawing and re-freezing up to three times. This plasma was used to assay progesterone and 17α-hydroxyprogesterone at dilutions of 1 in 500 and 1 in 1000 respectively.

4 μCi of $\left[1,2 - {}^{3}H\right]$ corticosterone were pipetted into a 100 ml standard flask and the solvent removed by evaporation under nitrogen. About 50 ml of tris - EDTA buffer, pH 8.0 were added and the flask was incubated 45°C for five minutes, the appropriate amount of plasma was then added (0.2 ml for the progesterone SPS and 0.1 ml for the 17α-hydroxyprogesterone SPS)

and the solutions were made up to 100 ml with buffer. The solutions were equilibrated at $4^{o}C$ overnight before use. These solutions could be stored for at least four weeks at $4^{o}C$ without reduction in their binding capacity.

2. Radioimmunoassay

0.05 M - phosphate buffer, pH 7.0, containing 0.01% (w/v) thimerosal, was prepared from di-sodium hydrogen orthophosphate and sodium dihydrogen orthophosphate in de-ionized, distilled water.

Gelatine containing dextran coated charcoal (0.125% charcoal, 0.025% dextran, and 0.100% gelatine), 0.5% gelatine solution and 0.1% gelatine solution were prepared in the buffer.

A stock solution of oestradiol (1ng/ml) was prepared in methanol. This solution was freshly prepared every four weeks as deterioration occurred on storage.

A solution of $\left[6,7\text{-}^{3}H\right]$ oestradiol containing approximately 25,000 dpm in 100 µl of solution was prepared in 0.1% gelatine solution.

An antiserum raised to oestradiol-17β-6(O-carboxymethyl)oxime-bovine serum albumin (Dean et al., 1971; Exley et al., 1971), a

gift from Dr. D. Exley, was used to assay oestradiol. The antiserum was obtained as a 1 in 5 dilution following Rivanol treatment and was stored in 0.05 ml aliquots at -20°C. Its properties were not affected by thawing and re-freezing or by storage for at least a year at -20°C. 0.01 ml of the antiserum was diluted to 50 ml with 0.1% gelatine solution, giving a 1 in 25,000 dilution of the original serum. This solution could be stored for up to four weeks at 4°C without appreciable reduction in binding capacity.

Measurement of Cortisol

Cortisol was assayed by the competitive protein binding method of Murphy (1967) under the supervision of Dr. R. Fraser, M.R.C. Blood Pressure Research Unit, Western Infirmary, Glasgow.

Measurement of Luteinizing hormone (LH)

In most cases LH was determined by radioimmunoassay based on the method of Greenwood et al., (1963), using an antiserum prepared against human chorionic gonadotrophin. The Medical Research Council Human Pituitary LH reference preparation 48/50 was used as the LH standard. These estimations were carried out by Mr. Crawford Ferguson, Research Department, the Royal Maternity Hospital, Glasgow.

In two cycles presented in Chapter 4 (Subject K.B., cycle 1, Fig. 4.VII and subject M.F., Fig. 4.VIII) LH was determined by radioimmunoassay using the 2nd IRP of HCG as the standard. These estimations were carried out under the supervision of Dr. J.W. Crawford, Department of Obstetrics and Gynaecology, University of Dundee.

Measurement of Follicle Stimulating hormone (FSH)

FSH was determined by radioimmunoassay using an antiserum prepared against FSH. The World Health Organisation International Reference Preparation of Human Menopausal Gonadotrophins (FSH and ICSH), 2nd IRP established 1964, was used as the FSH standard. These estimations were carried out by Searle Scientific Services, High Wycombe, under the supervision of Mr. Alan Craig.

CHAPTER 3

DEVELOPMENT OF COMPETITIVE PROTEIN BINDING AND RADIOIMMUNOASSAY METHODS FOR MEASUREMENT OF STEROID HORMONES

DEVELOPMENT OF COMPETITIVE PROTEIN BINDING (CPB) AND RADIOIMMUNOASSAY (RIA) METHODS FOR MEASUREMENT OF STEROID HORMONES

INTRODUCTION

Until 1964 only bioassays and chemical assays requiring large volumes of plasma or urine because of their insensitivity were available for measurement of steroid hormones. Murphy (1964) suggested ... the general application of displacement analysis, and in particular competitive protein binding, to the assay of small quantities of steroid hormones in biological fluids.

CPB analysis utilises the fact that many hormones *in vivo* are strongly bound to naturally occurring proteins. Where the hormone is antigenic, or may be rendered antigenic, it is possible to produce antibodies to the hormone of some degree of specificity. Utilisation of these proteins in displacement analysis is radioimmunoassay. The first substance to be measured by RIA was insulin by Berson and Yalow (1957). Unger et al., (1961) used the technique to determine glucagon and Hunter and Greenwood, (1962) reported the measurement of growth hormone by RIA. The principle of CPB has been applied to the measurement of much

smaller molecules than these such as thyroxine (Murphy and Pattee, 1964a) and vitamin B_{12} (Barakat and Ekins, 1961). Cortisol was the first steroid to be measured by this method (Murphy et al., 1963; Murphy and Pattee, 1964b).

PRINCIPLE OF DISPLACEMENT ANALYSIS

Displacement analysis depends upon the competition of two forms of the same molecule for reactive sites on a second molecule of lower concentration. One of the forms of the compound being assayed (a radio-active isomer) remains constant in amount while the other varies. Displacement of the isomer by increasing amounts of the variable, non-radioactive form allows the construction of a standard curve.

In applying this principle to CPB and RIA of steroids, one form of the steroid (the radioactive isomer) must first bind, with high affinity, to a limited amount of its specific binding protein in such a way that the binding sites on the protein are just saturated. If unlabelled steroid is then added to the system it will compete for the same binding sites so that the amount of radioactive steroid bound to the protein will be reduced due to its displacement by the unlabelled form. This displacement will be dependent upon the weight of unlabelled steroid added. Separation of free and protein bound labelled steroid

and quantization of either allows estimation of the degree of displacement of labelled steroid. Using standards of increasing weight, a standard curve to which unknown samples may be related can be constructed.

Application of these principles to the assay of steroid hormones in plasma is dependent upon the fulfilment of several important criteria. These are :-

1. Extraction of the steroid of interest from plasma.

2. Purification of the steroid; the amount of purification required is dependent upon the specificity of the binding solution.

3. Determination of the recovery of the steroid through the extraction and purification procedures.

4. High affinity binding of the steroid to a specific binding solution.

5. Availability of the steroid labelled with a radioactive isotope in high specific activity.

6. Separation of free and protein bound steroid.

7. Optimum incubation times and temperatures to achieve binding of radioactive steroid to protein, displacement of radioactive steroid by 'cold' steroid and separation of free and protein-bound steroid.

8. Measurement of the distribution of the steroid between free and bound fractions.

9. Comparison of unknown samples with a standard curve and calculation of results, with recovery corrections.

10. Assessment of methodological interfering factors.

11. Assessment of accuracy, precision and sensitivity.

The way in which these criteria have been applied to the measurement of progesterone, 17α-hydroxyprogesterone and oestradiol in plasma will be described in the following sections.

A. PROGESTERONE AND 17α-HYDROXYPROGESTERONE

Antoniades (1961) suggested that hormones are transported

in the blood in association with specific carrier proteins. Such proteins might play an important part in the transport of substances by increasing their solubility in plasma and by preventing their loss through the kidney. Murphy (1964) suggested the use of the protein CBG, an α-globulin present in all vertebrate species examined (Seal and Doe, 1966), for the measurement of a number of steroids, including progesterone, because of its ability to bind these steroids in a reversible manner. Many CPB methods developed for the measurement of progesterone and 17α-hydroxyprogesterone have utilised this protein (Neill et al., 1967; Yoshimi and Lipsett, 1968; Johansson, 1969; Martin et al., 1970). In the present study, the method of Martin et al., (1970) for progesterone was modified to make it quicker and more reliable and extended to combine the estimation of both progesterone and 17α-hydroxyprogesterone in the same sample of plasma.

1. Extraction of steroids from plasma. Extraction of the plasma with an organic solvent precipitated the protein, including any steroid binding globulin, which was present. Diethyl ether was found to be superior to ethyl acetate (Martin et al., 1970) as an extracting solvent firstly because of the efficiency of the steroid extraction (99% $\pm$ 3% SD., n = 200, of the progesterone and 17α-hydroxyprogesterone

were extracted in one extraction step) and secondly because of ease of evaporation. Decanting off the ether layer after freezing the aqueous layer in solid CO_2 gave a reproducibly more efficient extraction than did removal of the ether layer with a pasteur pipette.

2. Purification of Steroids. CBG is capable of binding all 4-ene, 3-one steroids to a greater or lesser extent, thus necessitating some purification of the steroids which were to be measured.

Paper partition chromatography was described in theory by Martin and Synge (1941) and solvent systems such as those described by Bush (1952) have proved most successful in separation, and purification of steroid hormones. A 'Bush-type' system utilising an aqueous stationary phase was developed in the present study for the separation and partial purification of progesterone and 17α-hydroxyprogesterone. The R_F values of various steroids in this system, light petroleum (boiling range 60^o - 80^oC) -methanol-water (10:7:3, v/v) are shown in Table 3.1. A band from R_F 0.05 to 0.15 was consistently removed for 17α-hydroxyprogesterone and a band from R_F 0.55 to 0.70 for progesterone.

17α-hydroxyprogesterone was not adequately separated

TABLE 3.1 R_F VALUES OF STEROIDS IN THE SYSTEM LIGHT PETROLEUM (BOILING RANGE 60°-80°C)-METHANOL-WATER (10:7:3, v/v)

STEROID	R_F	STEROID	R_F
PROGESTERONE	0.65	17α-HYDROXYPREGNENOLONE	0
17α-HYDROXYPROGESTERONE	0.07	16α-HYDROXYPREGNENOLONE	0
11α-HYDROXYPROGESTERONE	0.04	20α-HYDROXYPREGNENOLONE	0
20α-HYDROXYPROGESTERONE	0.23	20β-HYDROXYPREGNENOLONE	0
20β-HYDROXYPROGESTERONE	0.45	DHA	0.27
6β-HYDROXYPROGESTERONE	0.02	16α-HYDROXY DHA	0
16α-HYDROXYPROGESTERONE	0.01	PREGNANOLONE	0.89
TESTOSTERONE	0.10	PREGNANEDIONE	0.94
ANDROSTENEDIONE	0.41	PREGNANEDIOL	0
OESTRONE	0.13	OESTRONE ACETATE	0.86
OESTRADIOL	0.03	OESTRADIOL ACETATE	0.89
OESTRIOL	0	OESTRIOL ACETATE	0.80
ANDROSTENEDIOL	0	TESTOSTERONE ACETATE	0.85
CORTISOL	0.02	ANDROSTENEDIOL ACETATE	0.79
CORTICOSTERONE	0.03	11α-HYDROXYPROGESTERONE ACETATE	0.20
11 -DE OXYCORTICOSTERONE	0.13	6β-HYDROXYPROGESTERONE ACETATE	0.60
PREGNENOLONE	0.46	CORTISOL ACETATE	0.02

from testosterone by a single chromatography step in the above system (Table 3.1) and as this compound might interfere in the CPB assay the 17α-hydroxyprogesterone must be separated from it. Separation of the two steroids was achieved following acetylation. Testosterone acetylated under mild conditions whilst the 17α-hydroxyprogesterone did not, presumably because of the 'protective' effect of the side chain at the 17-position. An acetylating mixture consisting of 1 drop of pyridine and 1 drop of acetic anhydride was used and suitable conditions of time and temperature for complete acetylation of testosterone were investigated. The results of these investigations are shown in Table 3.2.

Two sets of conditions gave complete acetylation of testosterone without acetylation of 17α-hydroxyprogesterone, incubation for 5 h at room temperature or for 18 h at 4°C. The former was chosen as the more convenient for incorporation into the assay. The addition of 1 ml of methanol after the acetylation assisted in the removal of the excess reagents.

The testosterone acetate thus produced ran to an R_F value of 0.85 when rechromatographed in the same system and was thus well separated from the unchanged 17α-hydroxyprogesterone.

TABLE 3. 2 RESULTS OBTAINED FOLLOWING ACETYLATION OF A MIXTURE OF TESTOSTERONE (T) AND 17α-HYDROXY-PROGESTERONE (17 OHP) WITH ONE DROP OF PYRIDINE AND ONE DROP OF ACETIC ANHYDRIDE FOR VARIOUS TIMES AND AT DIFFERENT TEMPERATURES

(+ indicates presence of compound)
(- indicates absence of compound)

TIME h	TEMP °C	CONSTITUENTS OF ACETYLATED MIXTURE			
		T	T ACETATE	17 OHP	17 OHP ACETATE
1	4	+	+	+	-
	20	+	+	+	-
2	4	+	+	+	-
	20	+	+	+	-
5	4	+	+	+	-
	20	-	+	+	-
18	4	-	+	+	-
	20	-	+	+	+

3. Recovery of $[^3H]$ progesterone and $[^3H]$ 17α-hydroxyprogesterone. Solutions of $[^3H]$ progesterone and $[^3H]$ 17α-hydroxyprogesterone in benzene-ethanol (9:1, v/v) (10 µl; approximately 6,000 dpm; less than 0.02 ng of each) were added to the plasma prior to extraction. These internal standards were used to monitor recovery of the steroids throughout the extraction and purification procedures. Recoveries of 81.1 ± 7.5% S.D.,(n = 200) for progesterone and 54.9 ± 7.9% S.D., (n = 200) for 17α-hydroxyprogesterone were obtained.

4. The steroid binding solution.

a) Source of a suitable steroid binding protein. CBG has been shown to increase in concentration during pregnancy or during oestrogen administration (DeMoor et al., 1962). In the present study the administration of ethinyl oestradiol to a post-menopausal woman was used to produce plasma with a high concentration of this specific binding protein. The administration of dexamethasone was used to suppress the adrenal glands, to prevent saturation of the binding globulin with adrenal steroids (Lipsett et al., 1970).

The CBG used by Martin et al., (1970) was obtained from

dogs treated with ethinyl oestradiol and megestrol acetate. One of the most crucial points in the method was the production of a sufficiently sensitive CBG. Mayes and Nugent (1968) and Reeves et al., (1970) noted that in order to obtain sensitivity, precision and accuracy at 1.0 ng and below (which is required in the assay of 17α-hydroxyprogesterone in 1 ml of plasma) the binding protein must show a 25% change in bound radioactive isomer for 1 ng of 'cold' steroid added. Dog CBG will not yield a curve as sensitive as this, but this sensitivity was easily achieved with this human CBG. Reeves et al., (1970) have also shown that CBG from patients treated with oestrogen and dexamethasone appeared to be more sensitive than CBG from patients treated with oestrogen alone.

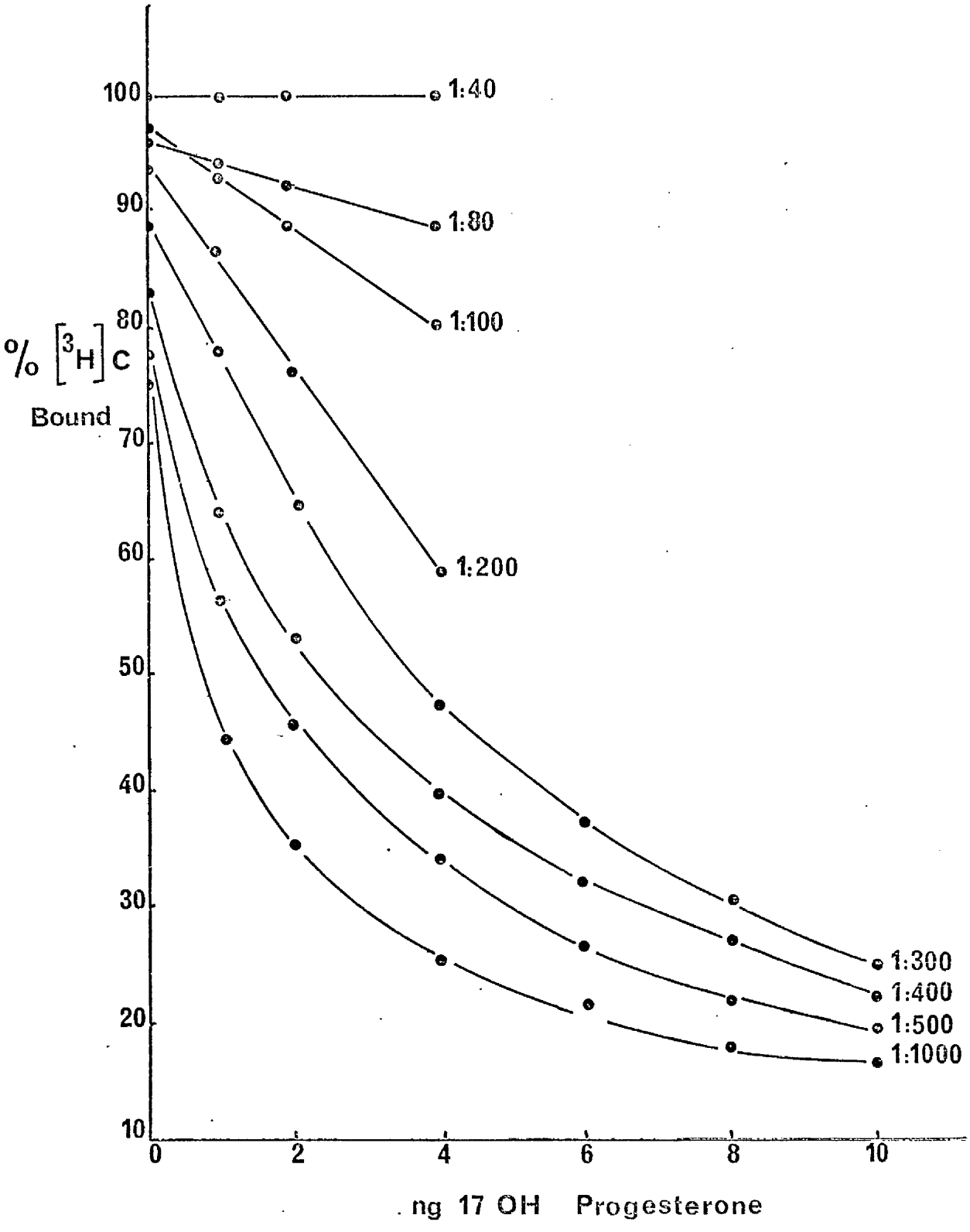
b) Preparation of the standard protein solution (SPS).

The binding of corticosterone to CBG is highly specific but steroids also bind to albumin. Although the latter binding is weak and non-specific it is nevertheless important because of the large concentration of albumin in plasma. However, albumin binding can be decreased by dilution of the plasma (Murphy, 1964). The determination of the

optimum dilution of plasma to obtain sensitivity over the required range for 17α-hydroxyprogesterone is shown in Fig. 3. I. At dilutions of 1 in 500 for progesterone and 1 in 1,000 for 17α-hydroxyprogesterone the albumin effect was neglible and maximum sensitivity was achieved through specific binding to a small concentration of CBG. A 1 in 500 dilution was chosen for assay of progesterone since this dilution gives a suitable initial binding and percentage displacement of $\left[^{3}H\right]$ steroid over the range 0 - 15 ng. A 1 in 1,000 dilution was chosen for the 17α-hydroxyprogesterone assay because this dilution gave a more sensitive curve over the range 0 - 4 ng.

Theoretically the CPB method relies on the ability of a steroid to displace its radioactive isomer from a binding protein. However, in practice steroids with the 4-ene, 3-one grouping vary in their binding affinity and hence in their ability to displace others from CBG (Reeves et al., 1970; Rossner, 1969). This explains the finding that better initial binding and displacement over the desired range was obtained when $\left[^{3}H\right]$ corticosterone was displaced by progesterone or 17α-hydroxyprogesterone than when $\left[^{3}H\right]$ progesterone or $\left[^{3}H\right]$ 17α-hydroxyprogesterone was used as the bound steroid. $\left[^{3}H\right]$ corticosterone was therefore

FIG. 3. I. DETERMINATION OF THE OPTIMUM DILUTION OF HUMAN POST-MENOPAUSAL PLASMA FOR THE MEASUREMENT OF 17α-HYDROXYPROGESTERONE. THE PERCENTAGE OF $[^3H]$ CORTICOSTERONE (C) BOUND IS PLOTTED AGAINST MASS OF 'COLD' 17α-HYDROXYPROGESTERONE (17 OH PROGESTERONE) FOR VARIOUS DILUTIONS OF PLASMA FROM 1 IN 40 TO 1 IN 1000



used in the preparation of the standard protein solutions. There will be further discussion of this phenomenon in Chapter 7.

Examples of the standard curves obtained at optimal dilutions for progesterone and 17α-hydroxyprogesterone are shown in Fig. 3. II and Fig. 3. III respectively.

c) Optimum pH for binding of $\left[^{3}H\right]$ corticosterone to CBG. During preliminary experiments on the binding of $\left[^{3}H\right]$ corticosterone to CBG it was observed that the percentage bound using a buffered solution at pH 8.0 was higher than that observed in deionized, distilled water (used by Martin et al., 1970 and Johansson, 1970). The results of further investigations of the effect of pH on binding are shown in Fig. 3. IV. The optimum binding occurred at pH 8.0. All subsequent work using CBG was performed in Tris-HCl-EDTA buffer at pH 8.0. De Moor et al., (1963) have also shown that the binding of corticosteroids to CBG is dependent on the pH of the solution, a maximum value having been observed at pH 8.0.

d) Specificity of the CBG. The 4-ene 3-one configuration in ring A of the steroid nucleus is essential for binding

FIG. 3. II A TYPICAL STANDARD CURVE FOR THE PROGESTERONE ASSAY OBTAINED USING A 1 IN 500 DILUTION OF HUMAN POST-MENOPAUSAL PLASMA. THE PERCENTAGE OF $[^3H]$ CORTICOSTERONE (C) BOUND IS PLOTTED AGAINST MASS OF 'COLD' PROGESTERONE

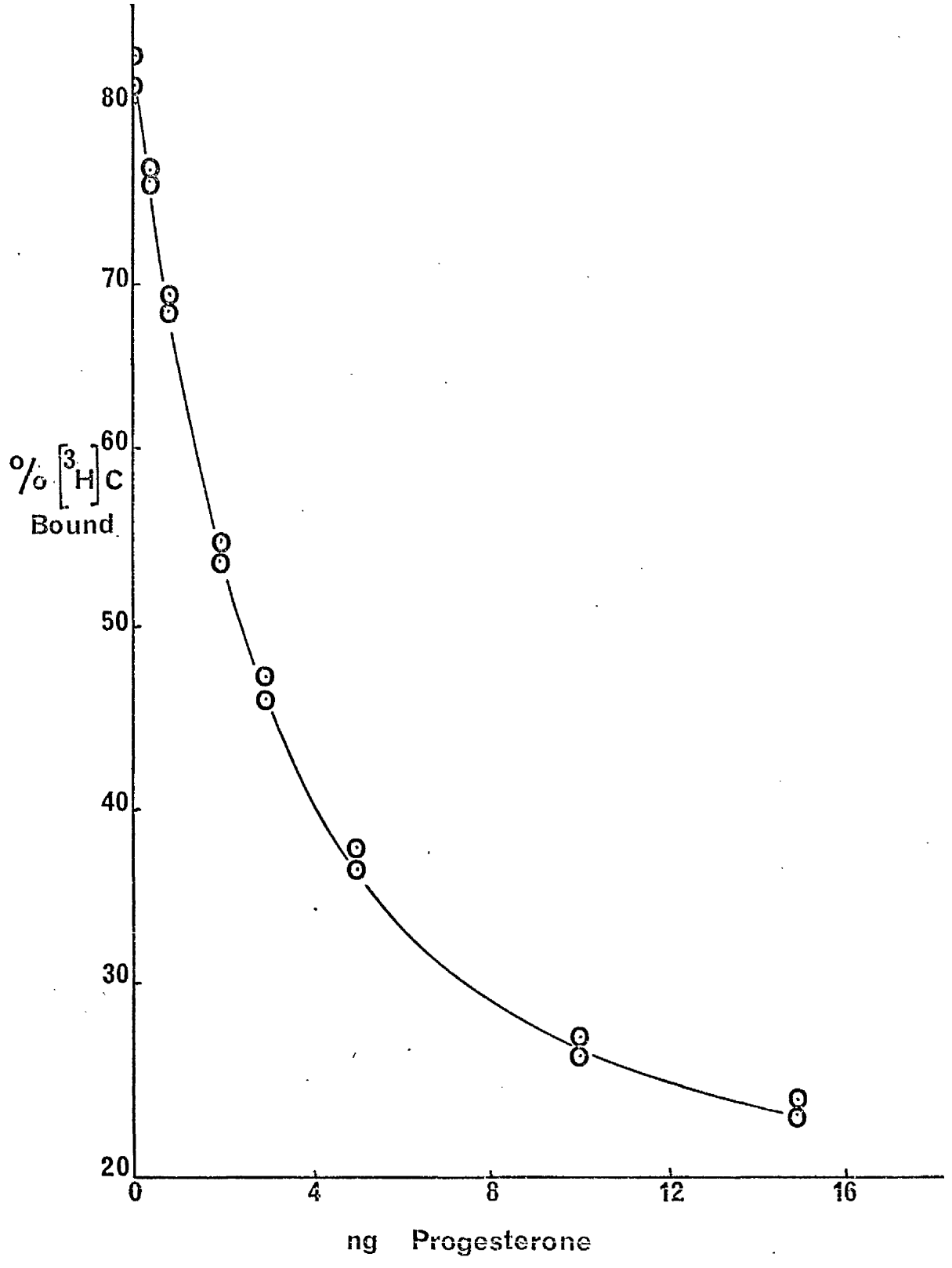


FIG. 3. III A TYPICAL STANDARD CURVE FOR THE 17α-HYDROXYPROGESTERONE ASSAY OBTAINED USING A 1 in 1000 DILUTION OF HUMAN POST-MENOPAUSAL PLASMA. THE PERCENTAGE OF $[^3H]$ CORTICOSTERONE (C) BOUND IS PLOTTED AGAINST MASS OF 'COLD' 17α-HYDROXYPROGESTERONE (17 OH PROGESTERONE)

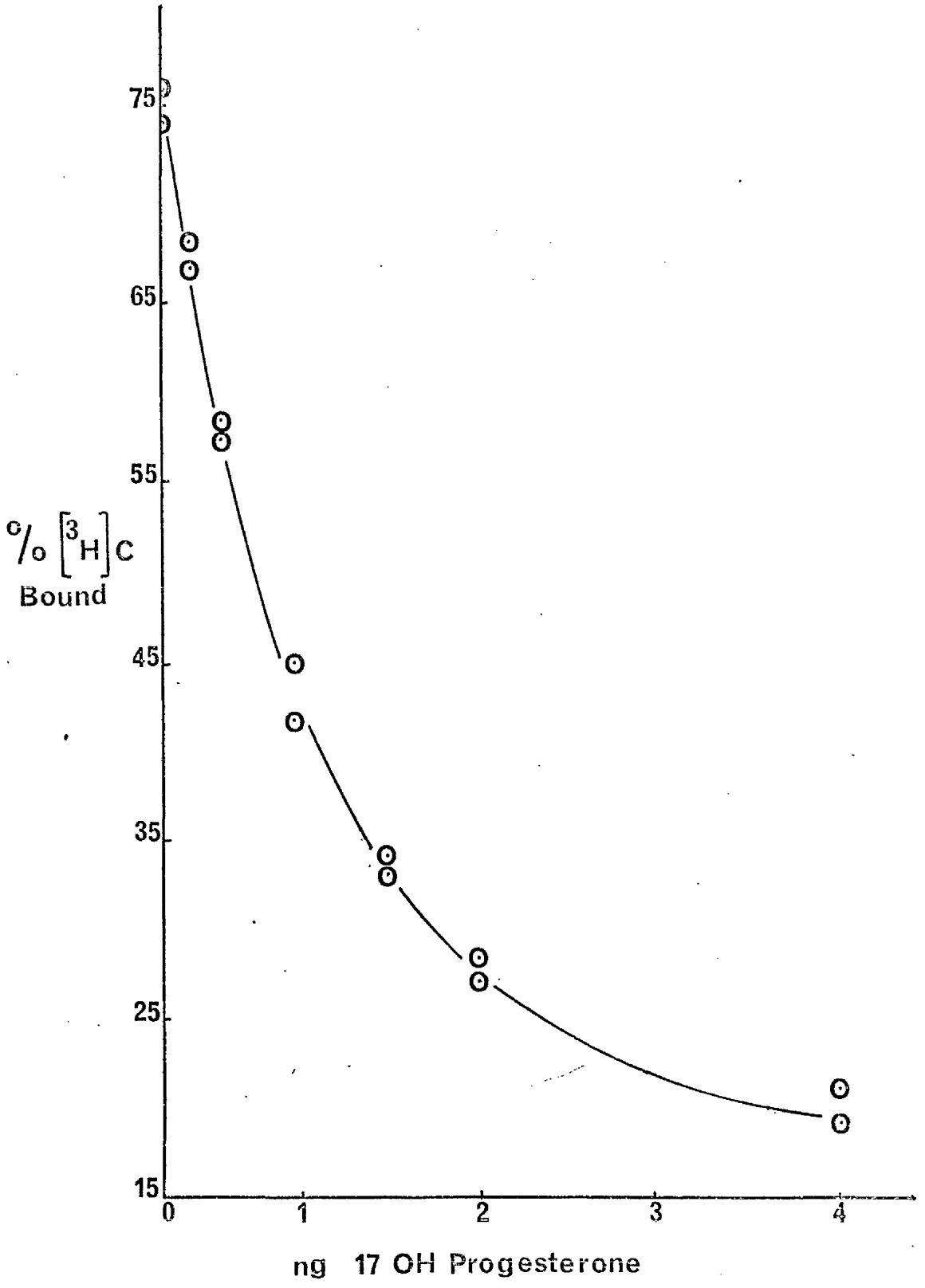
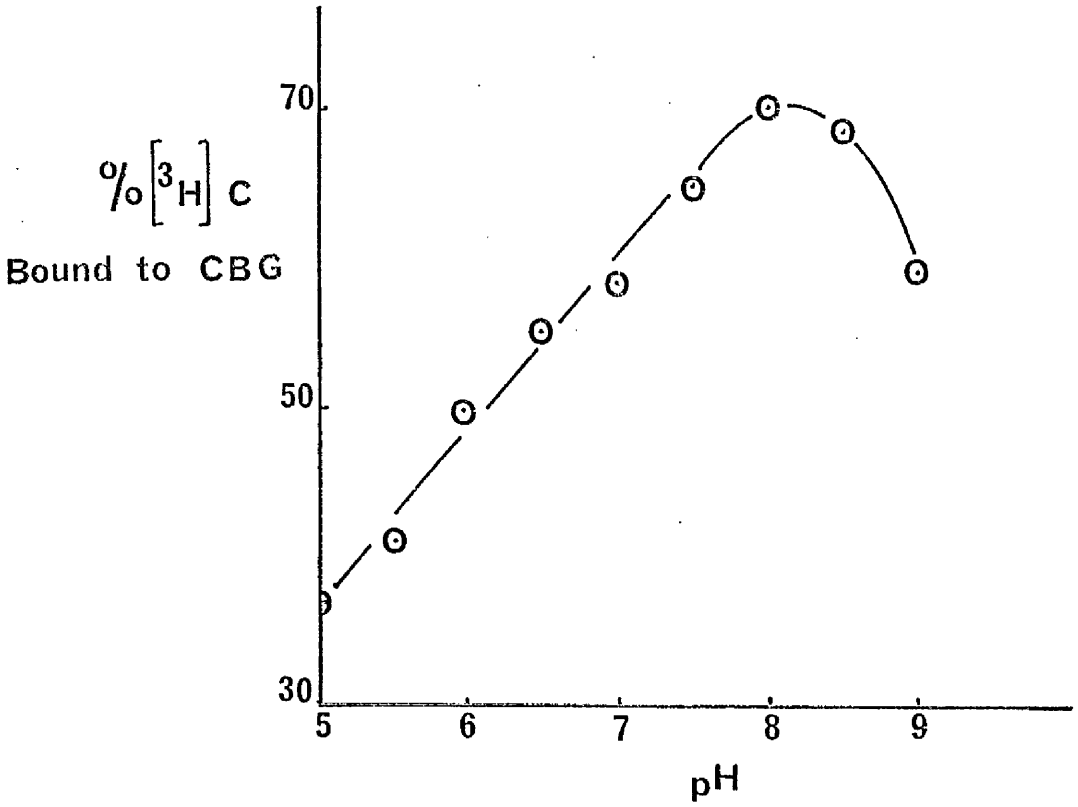


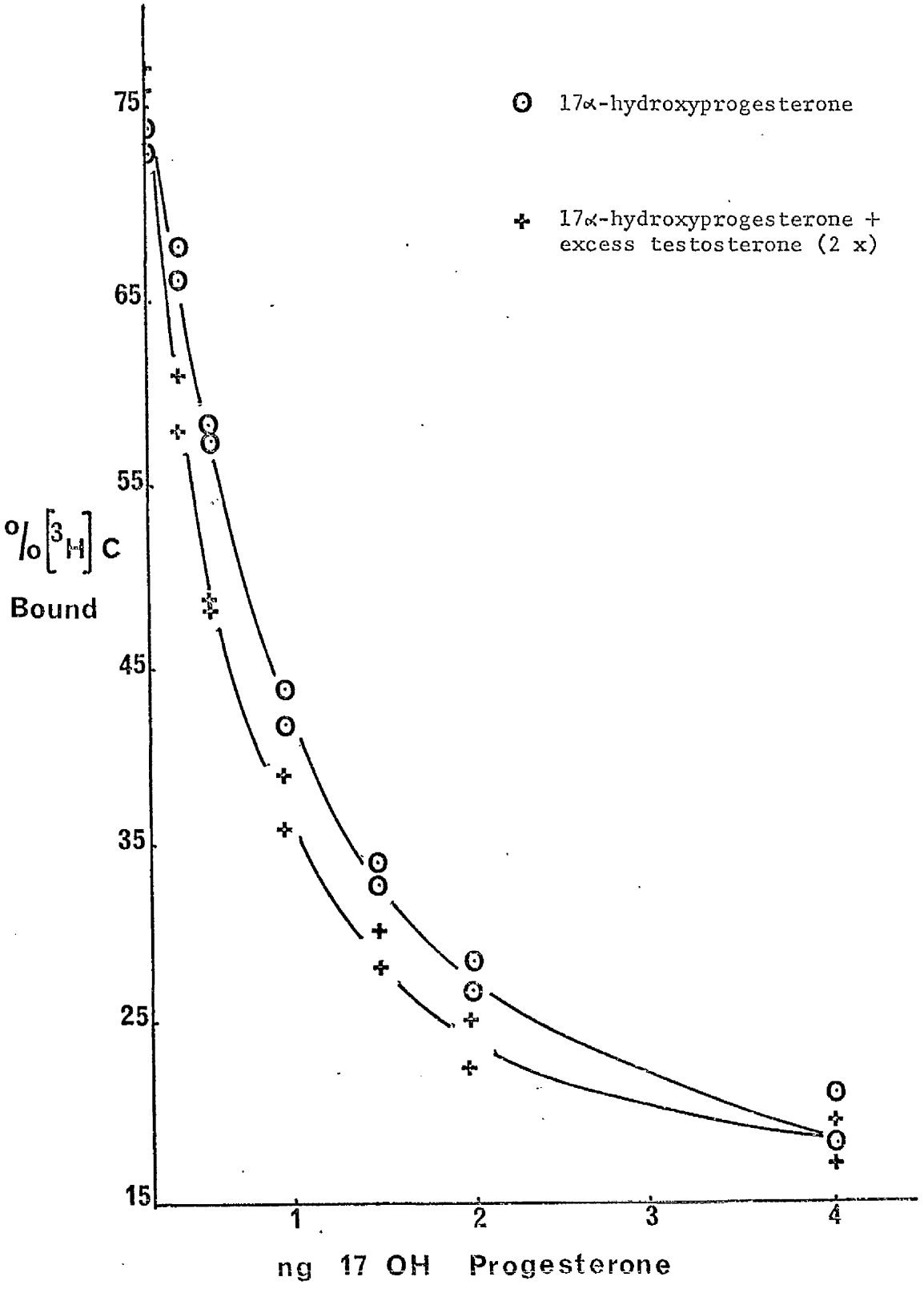
FIG. 3. IV THE EFFECT OF pH ON THE MAXIMUM BINDING OF $[^3H]$ CORTICOSTERONE (C) TO CBG. STANDARD PROTEIN SOLUTIONS (1 IN 1000 DILUTIONS) WERE PREPARED IN BUFFERS OF VARYING pH AND THE BINDING OF $[^3H]$ C TO THE CBG DETERMINED IN THE ABSENCE OF 'COLD' STEROID.



to CBG (Rossner, 1969; Sandberg et al., 1966; Reeves et al., 1970). Binding affinity is increased by the presence of hydroxyl groups in the 11β, 17α and 21 positions. The only steroid known to be present in human plasma which would compete for binding sites and would not be sufficiently separated from progesterone or 17α-hydroxyprogesterone in the initial chromatography is testosterone (see Table 3.1). Fig. 3.V. compares the standard curves obtained by displacement of $\left[^{3}H\right]$ corticosterone with 17α-hydroxyprogesterone alone and in the presence of testosterone. Testosterone increased the displacement of $\left[^{3}H\right]$ corticosterone and would therefore interfere in the assay of 17α-hydroxyprogesterone if it were not removed from the 17α-hydroxyprogesterone fraction.

5. Radioactive labelled steroid. In order to achieve sensitivity in the assay, the steroid used for initial binding must be labelled with a radioactive isotope of high specific activity, to ensure that the mass added was not large in relation to the amount of steroid being measured. Hence the $\left[^{3}H\right]$ steroid was used as $\left[^{14}C\right]$ steroids are of low specific activity.

FIG. 3. V COMPARISON OF STANDARD CURVES OBTAINED FOR 17α-HYDROXY-PROGESTERONE (17 OH PROGESTERONE) AND FOR 17α-HYDROXY-PROGESTERONE TO WHICH AN EXCESS (2 x) OF TESTOSTERONE WAS ADDED AT EACH POINT. THE PERCENTAGE OF $[^3H]$ CORTICOSTERONE (C) IS PLOTTED AGAINST MASS OF 'COLD' STEROIDS.



6. Separation of free and protein bound steroid. Various agents have been used to separate free and protein bound steroid in CPB assays including Sephadex (Kato and Horton, 1968), saturated ammonium sulphate solution (Mayes and Nugent, 1968), Florisil (Murphy, 1967; Frick and Kincl, 1969), Fuller's earth (Murphy, 1967) and dextran coated charcoal (Rosenfield et al., 1969). Martin et al., (1970) employed florisil in their assay but since publication of this work they have experienced difficulty in obtaining batches of florisil which will adequately reproduce this separation (Martin, 1970a). De Souza et al., (1970) and Strott and Lipsett (1968) have also reported similar problems with florisil. Dextran coated charcoal was therefore employed in the present study. This material, first used by Miller (1957), gave reproducible separation under the conditions of the assay. Addition of 0.5% gelatine solution prior to the dextran coated charcoal increased the percentage of $\left[^{3}H\right]$ corticosterone bound in the absence of 'cold' steroid by 10%. The role of the gelatine was uncertain but it appeared to give protection against 'stripping' of steroid bound to protein by the charcoal.

The dextran coated charcoal was stored at 4°C overnight before use and stirred continuously during use to ensure homogeneity of sampling in order to achieve good duplication.

7. Incubation times and temperatures. The incubation times and temperatures used in the CPB assay were the same as those used by Martin et al., (1970).

The SPS containing [^{3}H] corticosterone was incubated with the 'cold' steroid initially for 5 minutes at 45°C to obtain maximum dissociation of protein and [^{3}H] steroid and then at 4°C for 10 minutes. There is evidence that greater dissociation occurs between corticosterone and CBG at temperatures around 40°C (Westphal, 1970) while lowering the temperature to 4°C increases the association constants and thus enhances CBG binding to steroid (Murphy, 1964). In the second incubation, therefore, a new equilibrium was established in which [^{3}H] corticosterone was replaced on the CBG by increasing weights of progesterone or 17α-hydroxyprogesterone. After addition of dextran coated charcoal to separate free and protein bound steroid, the mixture was incubated for 10 minutes at 4°C. This was a suitable incubation time for obtaining

adsorption of free steroid without 'stripping' of the steroid bound to the protein.

8. Measurement of the distribution of steroid between free and bound fractions. After addition of dextran coated charcoal and incubation for 10 minutes at $4^{o}C$, the samples were centrifuged at 1,400 g for 10 minutes to precipitate the free steroid which had been adsorbed by the dextran coated charcoal. The steroid bound to protein remained in the supernatant. The percentage of $\left[^{3}H\right]$ corticosterone remaining bound to protein after displacement by 'cold' steroid was calculated by estimating the radioactivity in an aliquot of supernatant and comparison with the total $\left[^{3}H\right]$ corticosterone. This was determined by measuring the radioactivity in an aliquot of SPS in the absence of 'cold' steroid and dextran coated charcoal.

9. Comparison of unknown samples with a standard curve and calculation of results. A range of standard samples were prepared in duplicate for progesterone (0-15 ng) and 17α-hydroxyprogesterone (0 - 4 ng) from stock solutions in ethyl acetate (10 ng/ml, v/v) and the solvent evaporated.

The standard samples were processed in the CPB assay together with unknown samples. The percentage of $\left[^{3}H\right]$ corticosterone bound was determined, as described above, for the standard and unknown samples. Standard curves were constructed by plotting the percentage of $\left[^{3}H\right]$ corticosterone bound against corresponding weights of 'cold' steroid. The weights of steroid in unknown samples were determined by comparison with the standard curve. Results were expressed in ng/ml of plasma after correcting for procedural losses and volumes. Details of the calculation are presented in the Appendix (p.234).

10. Assessment of methodological interfering factors.

A suitable blank substance was assayed with each batch of samples to ensure that methodological interfering factors did not cause significant displacement of $\left[^{3}H\right]$ corticosterone from CBG. Choosing a suitable blank substance was complicated. Plasma from a gonadectomised, adrenalectomised female might have been of use but this had two main disadvantages. Firstly, such plasma was difficult to obtain in amounts sufficient for routine use. Secondly, it was possible that such drastic treatment might have altered the composition of the plasma, thus invalidating

the blank.

Charcoal treated plasma has been used (Lipsett et al., 1970) but it cannot be claimed that this is equivalent to steroid-free plasma as, in addition to removing steroids, charcoal adsorbs many other low molecular weight substances which may contribute to blank values.

A third possible blank substance was water, which was used in this study. This was not the perfect choice but gave an estimate of interference due to methodology.

The blank values obtained for 5 ml of deionized, distilled water were 0.02 $\pm$ 0.05 ng S.D. (n = 56) for progesterone and 0.02 $\pm$ 0.03 ng S.D. (n = 56) for 17α-hydroxyprogesterone.

11. Statistical assessment of the method.

a) Accuracy. Ten replicate samples each of 17α-hydroxyprogesterone (2 ng) and progesterone (2 ng, 5 ng and 8 ng) were added to blanks and assayed. The results obtained are shown in Table 3.3. Accuracies of 96.5% for 2 ng of 17α-hydroxyprogesterone and 106% (2 ng), 100% (5 ng) and 104% (8 ng) for progesterone were obtained.

b) Precision. From the figures given in Table 3.3, the intra-assay coefficient of variation for 2 ng of 17α-

hydroxyprogesterone was ± 8.8% and those for progesterone were ± 11.8%, ± 10.4% and ± 5.5% for 2 ng, 5 ng, and 8 ng, respectively.

The inter-assay coefficient of variation was determined by assaying, in duplicate, a quality control plasma sample with each assay. The results for these samples were 1.62 ± 0.16 ng S.D. (n = 50) for 17α-hydroxyprogesterone and 4.0 ± 0.46 ng S.D. (n = 50) for progesterone. These figures gave an inter-assay coefficient of variation at these levels of ± 9.9% for 17α-hydroxyprogesterone and ± 11.5% for progesterone.

TABLE 3.3 RESULTS OF THE ASSAY OF 17α-HYDROXYPROGESTERONE AND PROGESTERONE ADDED TO BLANK SAMPLES

STEROID ADDED	WEIGHT ADDED (ng)	RESULT (ng ± S.D.)
17α-hydroxyprogesterone	2	1.93 ± 0.17
Progesterone	2	2.12 ± 0.25
Progesterone	5	5.00 ± 0.52
Progesterone	8	8.30 ± 0.46

c) Sensitivity. The blank value plus one S.D. was used as an estimate of the lowest level of detection of the steroid. Hence from the figures given in section 10, (p. 46) the lowest level of detection of progesterone was 0.07 ng and of 17α-hydroxyprogesterone, 0.05 ng per sample.

The method finally developed for the assay of progesterone and 17α-hydroxyprogesterone, incorporating these criteria is summarised below.

Assay of progesterone and 17α-hydroxyprogesterone by CPB.

Solutions of $\left[^3H\right]$ progesterone and $\left[^3H\right]$ 17α-hydroxy-progesterone in benzene-ethanol (9:1 v/v) (10 μl; approximately 6,000 dpm, less than 0.02 ng of each) were added to the plasma to be assayed (1 ml) and the samples diluted to 5 ml with deionized, distilled water. The plasma was extracted by shaking with 5 ml of diethyl ether in stoppered tubes for 45 seconds. After allowing the layers to separate the tubes were placed in crushed solid CO_2; the aqueous layer was frozen rapidly and the ether layer decanted into conical tubes. The solvent was removed by evaporation under nitrogen, the tubes washed down with 1 ml of ether and again taken to dryness.

The plasma extracts were dissolved in a few drops of ethyl acetate and applied to pre-washed chromatography papers cut into lanes as previously described (p. 22). Standards of progesterone and 17α-hydroxyprogesterone (0.5 μCi) were applied to two lanes on each paper. The papers were chromatographed in a descending system of light petroleum (boiling range 60° - 80°C) - methanol-water (10:7:3, v/v) for three-and-a-half hours at room temperature, after equilibration overnight. They were removed from the tanks, dried and the standard lanes on each paper were scanned using the radiochromatogram scanner. The bands corresponding to progesterone and 17α-hydroxyprogesterone standards on each sample lane were cut out and eluted with 3 ml of methanol. The eluates were evaporated to dryness under nitrogen.

The 17α-hydroxyprogesterone dried eluates were acetylated with 1 drop of pyridine and 1 drop of acetic anhydride for 5 hours at room temperature. 1 ml of methanol was added and the reagents were removed by evaporation under nitrogen in a water bath at a temperature below 30°C. The acetylated 17α-hydroxyprogesterone bands were re-chromatographed in the same system and the 17α-hydroxyprogesterone, which was now separated from testosterone acetate was eluted from the paper with 3 ml of methanol and the solvent evaporated.

1 ml of ether was added to the dried eluates of both progesterone and 17α-hydroxyprogesterone, and 0.2 ml removed from each for radioactivity determination to monitor the recovery throughout the extraction and purification steps. The remaining 0.8 ml of ether was evaporated and the dried residues were assayed by CPB together with standard samples.

The range of standard samples was prepared in duplicate from 0 - 15 ng for progesterone and 0 - 4 ng for 17α-hydroxy-progesterone using 10 ng/ml solutions in ethyl acetate and the solvent evaporated under nitrogen.

Standard protein solution (1 ml) was added to all unknown samples and the standard samples and contents of the tubes were mixed and incubated at $4^{o}C$ for 5 minutes. The tubes contents were mixed again and the tubes put into an ice/water bath for 10 minutes. 0.5 ml of 0.5% gelatine solution and 0.5 ml of dextran coated charcoal suspension were added to each tube, except two tubes, referred to as 'no charcoal' tubes which contained no 'cold' steroid and 0.5 ml of buffer in place of the dextran coated charcoal. The tubes were shaken gently, left in ice for 10 minutes and then centrifuged at 1,400 g for 10 minutes. 0.5 ml of the supernatant was removed for radioactivity determination. Results were calculated as percentage $\left[^{3}H\right]$ corticosterone remaining bound after displacement by 'cold'

steroid using the 'no charcoal' tubes to estimate the total amount of $[^3H]$ corticosterone.

Blanks consisting of 5 ml of deionized, distilled water and quality control plasma samples were processed with each assay.

The extraction and purification steps and CPB assay are outlined in flow sheets (Fig. 3. VI and Fig. 3. VII).

B. OESTRADIOL

The most specific type of binding proteins, antibodies, were used in the assay of oestradiol. This hormone is present in non-pregnant human female plasma in sub-nanogram amounts per ml, necessitating a highly sensitive method for its measurement in small volumes of plasma. Radio immunoassay could provide this high level of sensitivity. The method developed was a modification of that described by Wu and Lundy (1971) using a different antiserum.

1. Extraction of oestradiol from plasma. Diethyl ether was found to be the most suitable extracting solvent giving recoveries of 100 $\pm$ 2% S.D. (n = 30) of $[^3H]$ oestradiol added to plasma. In the assay, no $[^3H]$

FIG. 3. VI FLOW SHEET OF THE EXTRACTION AND PURIFICATION OF PROGESTERONE (P) AND 17α-HYDROXYPROGESTERONE (17 OHP)

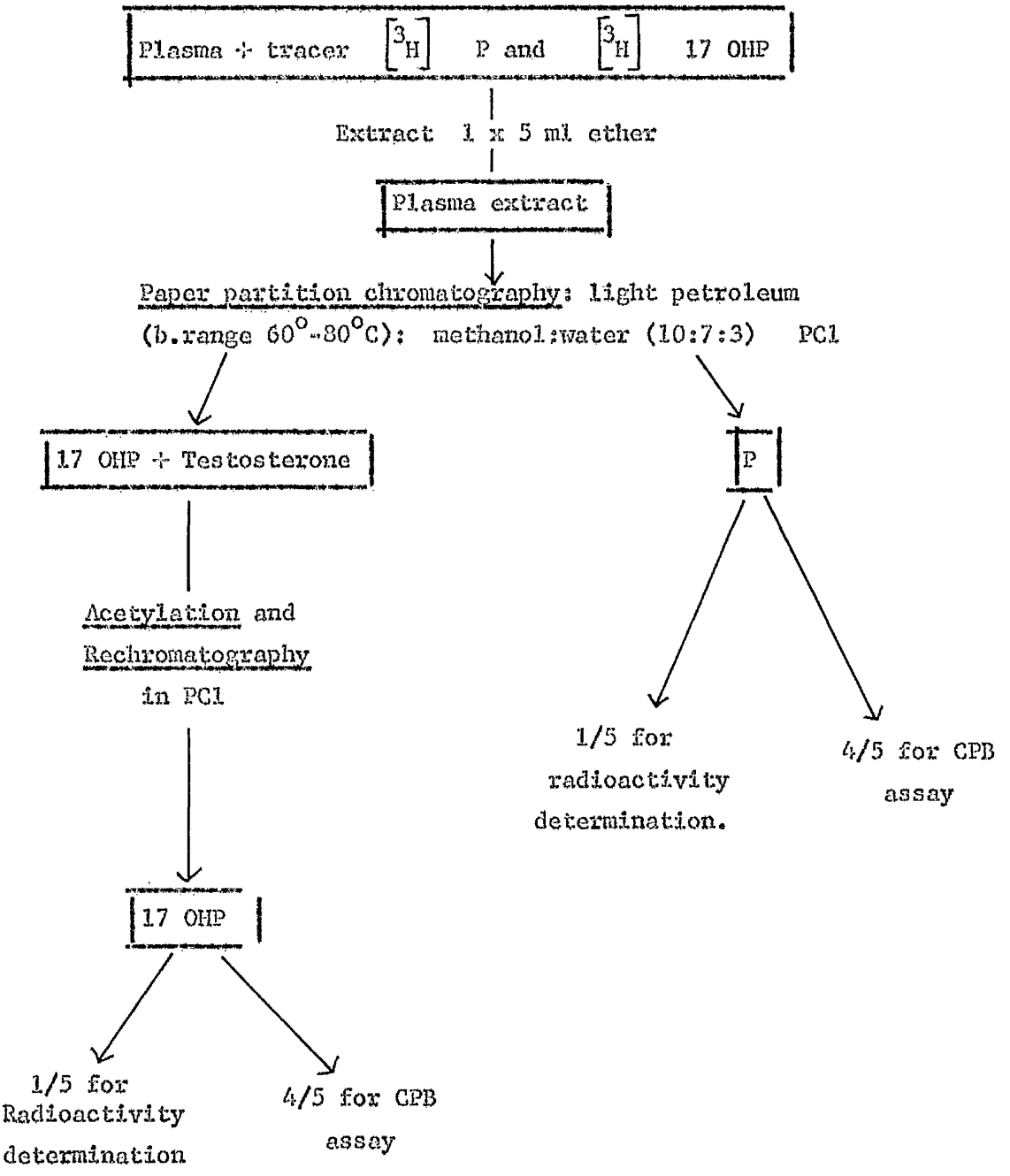
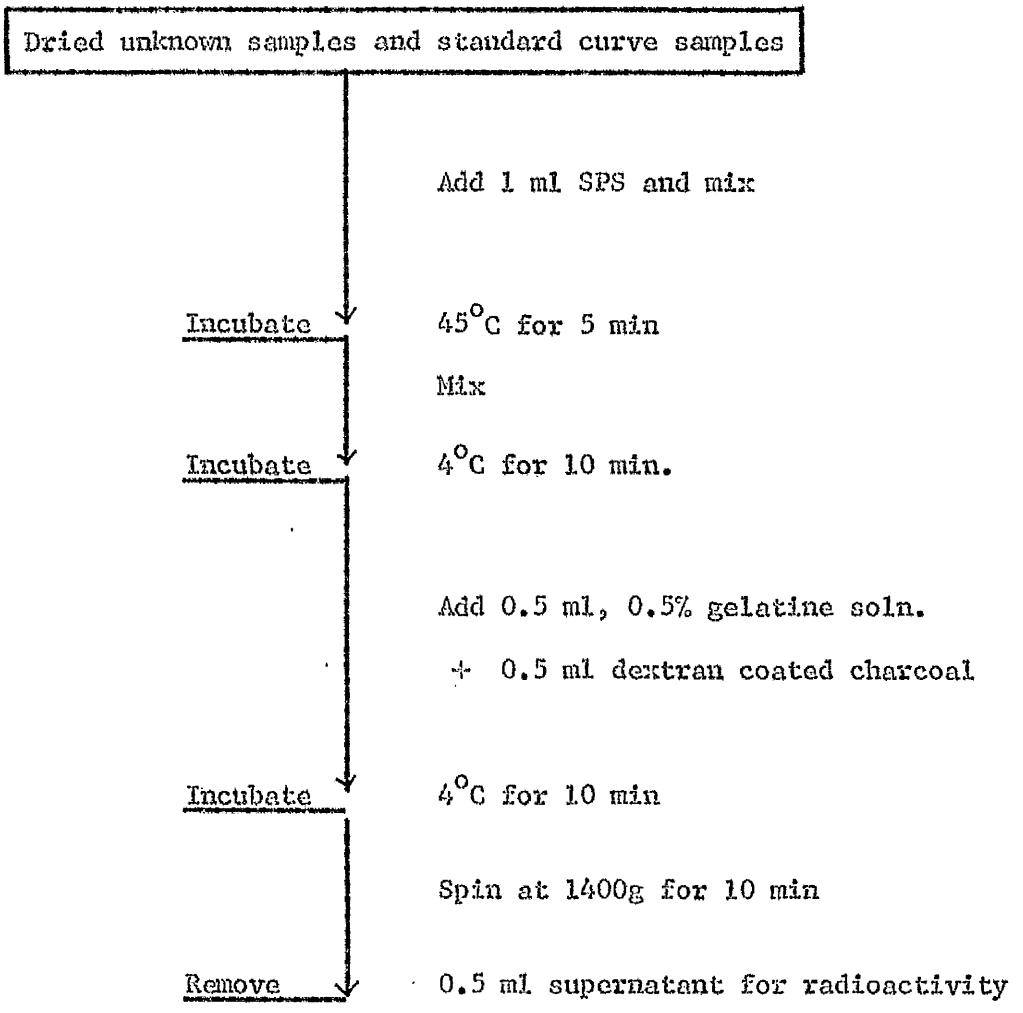


FIG. 3. VII FLOW SHEET OF CPB ASSAY FOR PROGESTERONE AND 17α-HYDROXYPROGESTERONE



tracer of oestradiol was added to the plasma as preliminary studies showed that the extraction was reproducible and quantitative.

2. Purification of oestradiol. As the antibody used was specific for oestradiol (see p. 55) it was theoretically possible to set up a specific assay without a chromatographic purification step. In preliminary experiments samples were assayed without chromatography and compared to identical samples subjected to chromatography on Sephadex LH20 (Wu and Lundy, 1971) to separate oestradiol from oestrone and oestriol. The results of these assays are shown in Table 3. 4. There is no significant difference between the results obtained by methods (i) and (ii) (paired t, 0.49, $0.7 > p > 0.6$) and therefore chromatography was not included in the assay procedure.

3. Recovery of $[^3H]$ oestradiol. As mentioned in section 1 above, no $[^3H]$ internal standard of oestradiol was added to the plasma, since the extraction was quantitative and no chromatography step was involved. Recovery was assumed to be 100% in all samples.

TABLE 3. 4 COMPARISON OF THE RESULTS OBTAINED FOR THE ASSAY OF OESTRADIOL IN NORMAL MENSTRUAL CYCLE PLASMA (i) WITHOUT CHROMATOGRAPHY AND (ii) WITH CHROMATOGRAPHY ON SEPHADEX LH 20

DAY OF CYCLE	(i) OESTRADIOL (pg/ml)	(ii) OESTRADIOL (pg/ml)
1	90	85
2	40	50
5	45	45
7	80	95
9	65	50
13	200	250
14	70	70
15	75	60
16	100	120
17	140	120
19	200	215
20	215	200
21	155	200
22	175	150
23	65	75
26	95	70
Mean $\pm$ S.D.	113.1 $\pm$ 58.8	115.1 $\pm$ 66.9

4. The oestradiol binding solution.

a) Production of a suitable antiserum. There have been many reports recently of the production of suitable antibodies for RIA of oestrogens (Ferin et al., 1968; Abraham, 1969; Mikhail et al., 1970; Dean et al., 1971). Since steroids alone are non-antigenic (Lieberman et al., 1959) it is necessary to attach the steroid to a large 'foreign' protein molecule such as bovine serum albumin (BSA) before injection into an animal. The point of conjugation of the steroid to BSA is important with regard to the specificity of the antiserum produced. Greatest specificity has been obtained for the steroid by attachment of the protein so that the functional groups of the steroid molecule are free. This promotes production of antibodies specific for these groups (Dean et al., 1971). Oestradiol antibodies of greater specificity have been established by linking the steroid to the BSA through the 6-position (Exley et al., 1971) than linking through the 17-position (Wu and Lundy, 1971) or through the 3-position (Thorneycroft et al., 1970), of the steroid nucleus.

A mixed population of antibodies is produced both to the steroid and BSA and the latter can be selectively

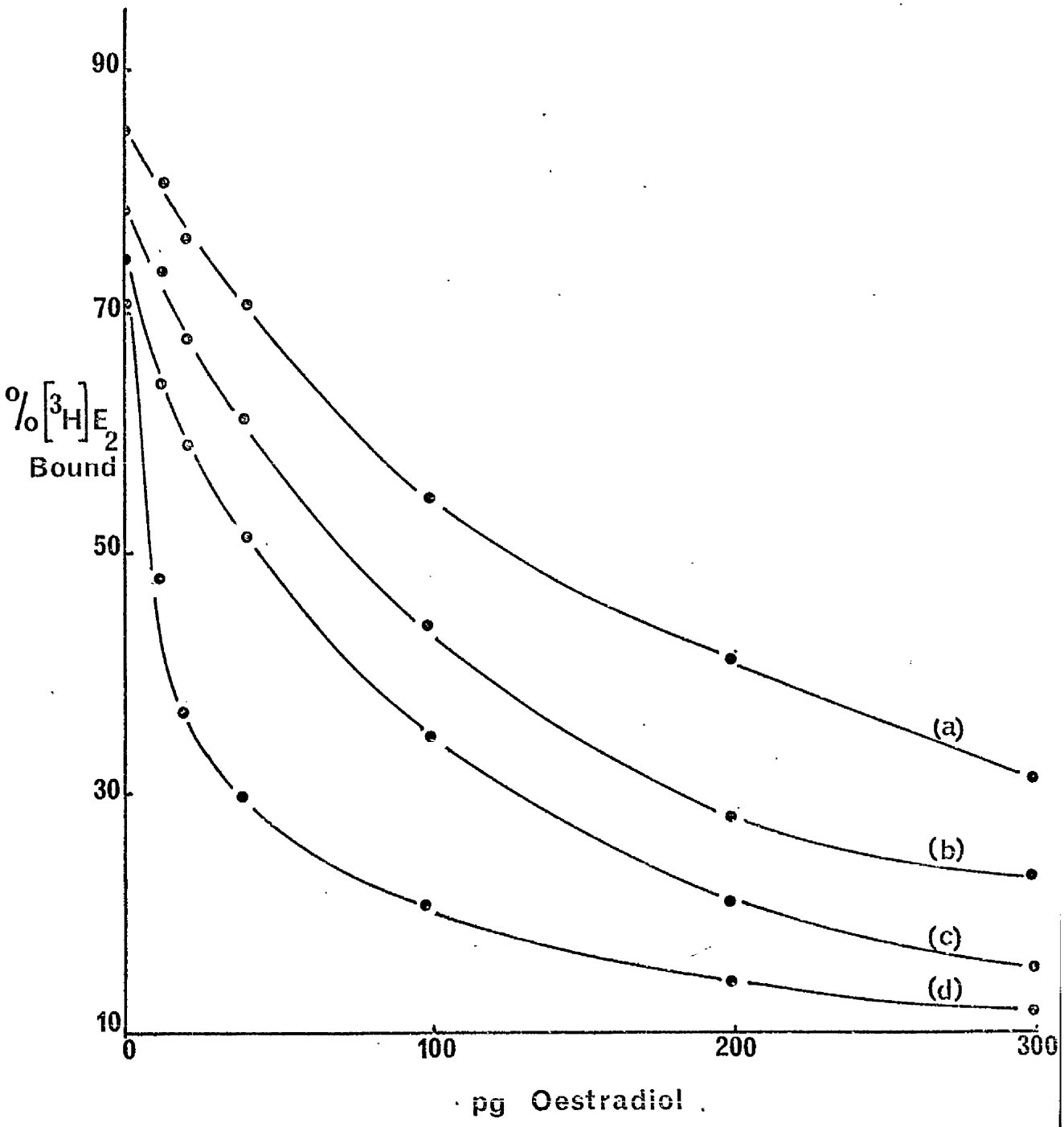
removed to produce a very high titre antiserum. Addition of BSA removes BSA antibodies (Campbell et al., 1964; Exley et al., 1971) whilst treatment with Rivanol (diamino lactate) can be used to remove serum proteins (albumin and α and β globulins) which might otherwise take part in non-specific binding (Horesji and Smetana, 1956; Abraham, 1969).

b) Optimum dilution of antiserum. The results of experiments to determine the optimum dilution of antiserum for use over the range 0 - 300 pg are shown in Fig. 3. VIII. 1 in 25,000 was chosen as a suitable dilution and was prepared in 0.05M-phosphate buffer, pH 7.0.

c) Specificity of antiserum. The cross reaction with other compounds was examined in the following way. Standard samples of oestradiol were prepared together with standard samples of the cross reacting substance and samples of oestradiol to which the cross reacting substance had been added. These three sets of standards were assayed together and the results compared. In the case of substances likely to be found in the plasma in greater

FIG. 3. VIII STANDARD CURVES OBTAINED FOR OESTRADIOL USING VARIOUS DILUTIONS OF ANTISERUM. THE PERCENTAGE OF $[^3H]$ OESTRADIOL (E_2) BOUND IS PLOTTED AGAINST MASS OF 'COLD' OESTRADIOL.

(a) 1 in 10,000
(b) 1 in 20,000
(c) 1 in 25,000
(d) 1 in 30,000



concentration than oestradiol, larger amounts of the test substance than oestradiol were added when the standards were prepared. Fig. 3. IX compares curves obtained for oestradiol, oestrone and oestriol. The results, indicated that oestrone and oestriol gave a small displacement of $\left[^{3}H\right]$ oestradiol from the antiserum when they were present alone. However, when oestrone and/or oestriol were added to oestradiol in equal concentration, the results shown in Fig. 3. X were obtained. The curves did not differ significantly, indicating that neither oestrone nor oestriol, when present with oestradiol, caused further displacement of $\left[^{3}H\right]$ oestradiol from the antiserum. Table 3. 5 shows the percentage cross reaction of various substances with the oestradiol antiserum. Calculation of the cross reaction was made using the formula of Abraham (1969):

FIG. 3. IX COMPARISON OF STANDARD CURVES OBTAINED FOR OESTRADIOL OESTRONE AND OESTRIOL WITH THE OESTRADIOL ANTISERUM (1 in 25,000 DILUTION). THE PERCENTAGE OF $[^3H]$ OESTRADIOL (E_2) BOUND IS PLOTTED AGAINST MASS OF 'COLD' OESTROGEN.

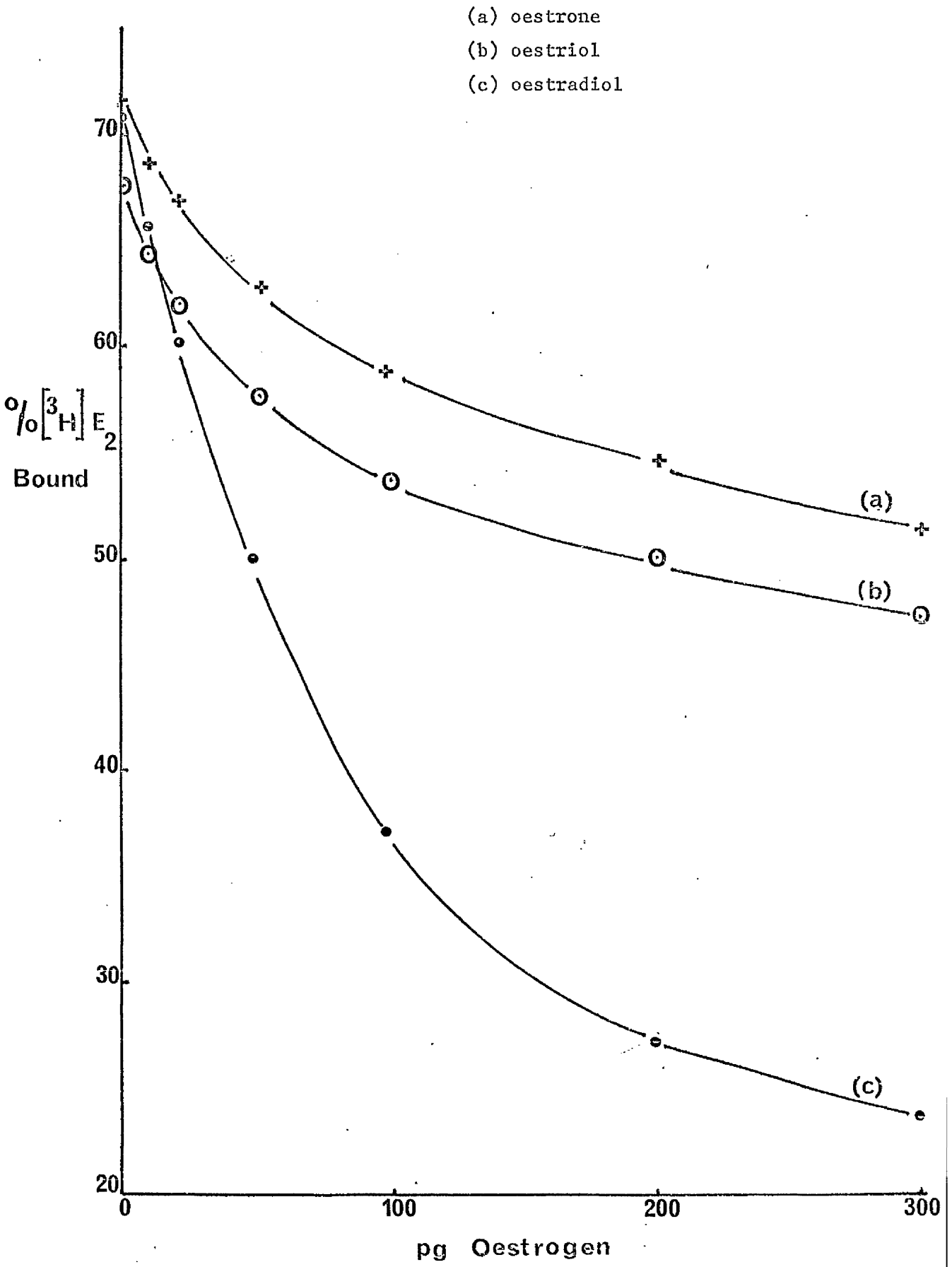


FIG. 3. X COMPARISON OF STANDARD CURVES OBTAINED FOR OESTRADIOL ALONE AND IN COMBINATION WITH OESTRONE AND/OR OESTRIOL, USING THE OESTRADIOL ANTISERUM. THE PERCENTAGE OF $[^3H]$ OESTRADIOL (E_2) BOUND IS PLOTTED AGAINST MASS OF 'COLD' OESTROGEN.

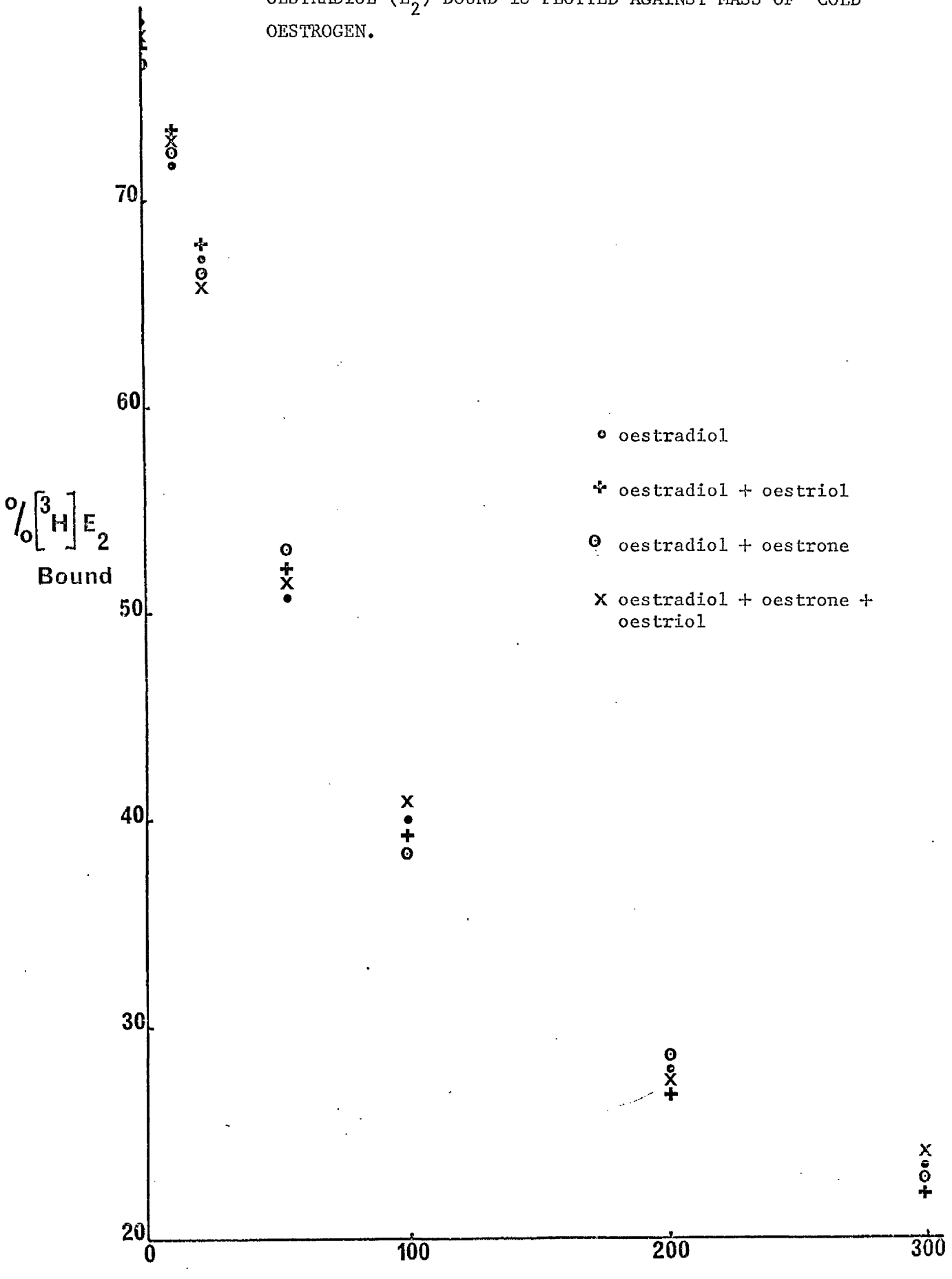


TABLE 3. 5 PERCENTAGE CROSS REACTION OF VARIOUS COMPOUNDS WITH OESTRADIOL ANTISERUM

COMPOUND	CROSS-REACTION (%)
* OESTRADIOL-17β	100
* OESTRIOL	1.0
* OESTRONE	0.9
+ 6-KETO-OESTRADIOL-17β	110
* TESTOSTERONE	< 0.01
* PROGESTERONE	< 0.01
* 17α-HYDROXYPROGESTERONE	< 0.01
+ OESTRADIOL-17α	2.0
* CLOMIPHENE CITRATE	0
+ DEHYDROEIPANDROSTERONE	< 0.01
+ CHOLESTEROL	< 0.01
+ CORTISOL	< 0.01

* Personally determined

+ Figures quoted from Exley et al., (1971)

Mass oestradiol required to displace 50% of bound $\left[{}^{3}H\right]$ oestradiol = x

Mass of cross reacting substance required to displace 50% of bound $\left[{}^{3}H\right]$ oestradiol = y

$$\% \text{ cross reaction} = \frac{x}{y} \times 100$$

The figures indicated that providing the concentration of oestrogenic compounds, substituted at the 6-position of the steroid nucleus in the sample to be assayed for oestradiol was low (Exley et al., 1971) the antiserum was sufficiently specific to be used for direct RIA on organic extracts of plasma samples.

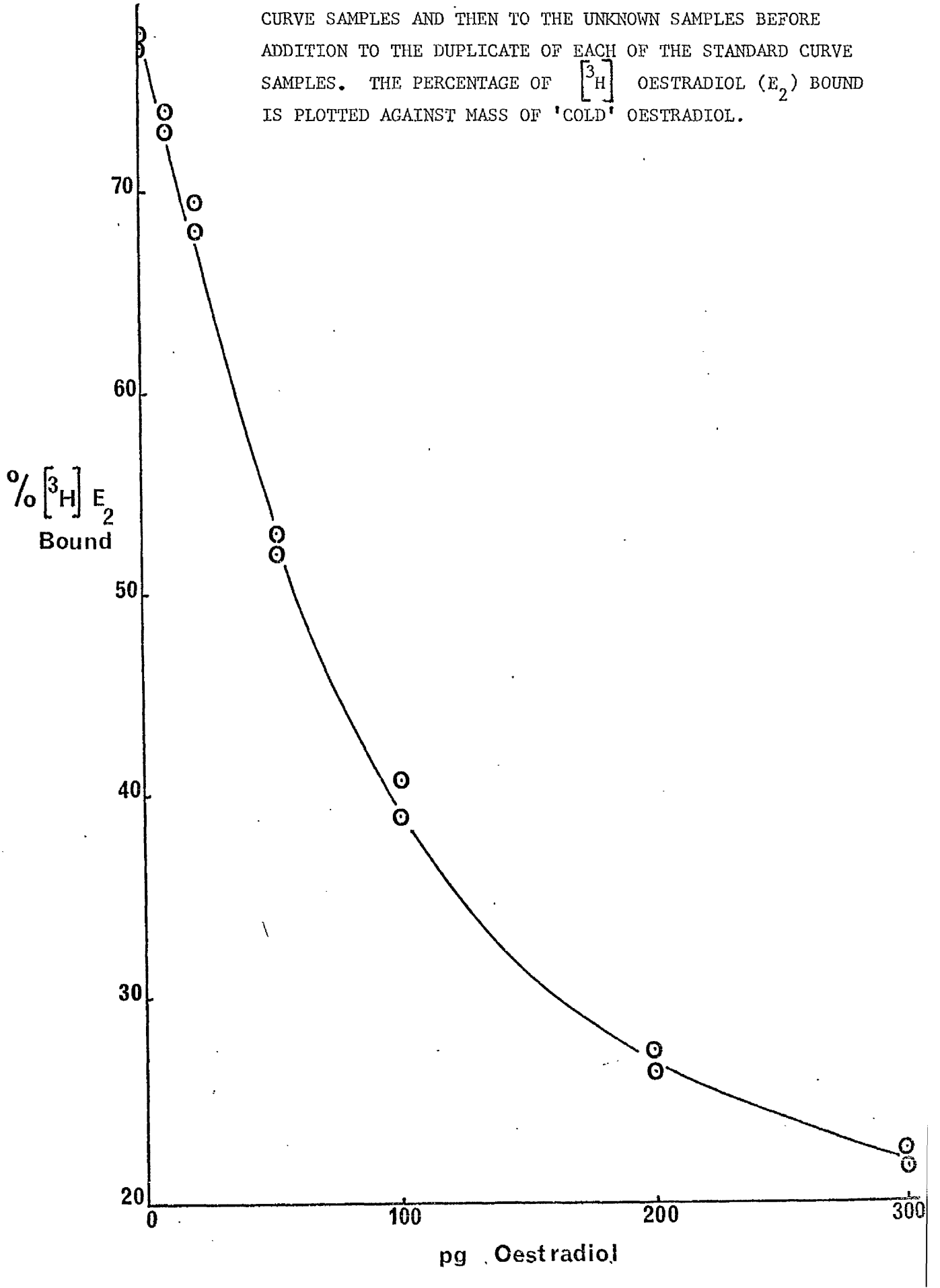
5. Radioactive labelled oestradiol. Exley et al., (1971), reported no difference between the binding of $\left[6,7\text{-}{}^{3}H\right]$ oestradiol and $\left[2,4,6,7,\text{-}{}^{3}H\right]$ oestradiol to this oestradiol antiserum. $\left[6,7\text{-}{}^{3}H\right]$ oestradiol was used in the present study.

6. Separation of free and protein bound oestradiol. Several methods have been reported for separation of free and antibody bound hormone in RIA. These include ammonium sulphate precipitation, (Chard et al., 1971) double antibody technique (Midgley, 1966), dry coating of plastic tubes

with antisera (Catt and Tregear, 1967) chromatoelectrophoresis (Saxena et al., 1968) and dextran coated charcoal (Wu and Lundy, 1971). Dextran coated charcoal was used in the present study. Separation of free and bound steroid was achieved successfully using half the concentration of charcoal described previously for the CPB assay.

The time of contact of charcoal with the RIA equilibrium mixture was found to be critical. Table 3.6 shows the apparent decrease in percentage $\left[{}^{3}H\right]$ oestradiol bound with increasing 'contact' time with charcoal. It was therefore necessary to centrifuge the tubes immediately after the addition of charcoal. This could give rise to problems of inconsistency between the first and the last additions if the number of tubes handled was large. To attempt to eliminate the errors involved in this procedure, in practice, the charcoal suspension was added to one of each of the standard curve samples, then to the unknown samples and finally to the duplicate of each of the standard curve samples. A typical standard curve, treated in an assay in this way, is shown in Fig. 3. XI. Duplication of the points was good, showing that little

FIG. 3. XI A TYPICAL STANDARD CURVE OBTAINED FOR THE OESTRADIOL ASSAY, USING A 1 IN 25,000 DILUTION OF THE ANTISERUM. IN OBTAINING THIS CURVE, THE DEXTRAN COATED CHARCOAL SUSPENSION WAS ADDED TO ONE OF EACH OF THE STANDARD CURVE SAMPLES AND THEN TO THE UNKNOWN SAMPLES BEFORE ADDITION TO THE DUPLICATE OF EACH OF THE STANDARD CURVE SAMPLES. THE PERCENTAGE OF $[^3H]$ OESTRADIOL (E_2) BOUND IS PLOTTED AGAINST MASS OF 'COLD' OESTRADIOL.



error was introduced in this procedure when up to thirty-six tubes were handled in an assay.

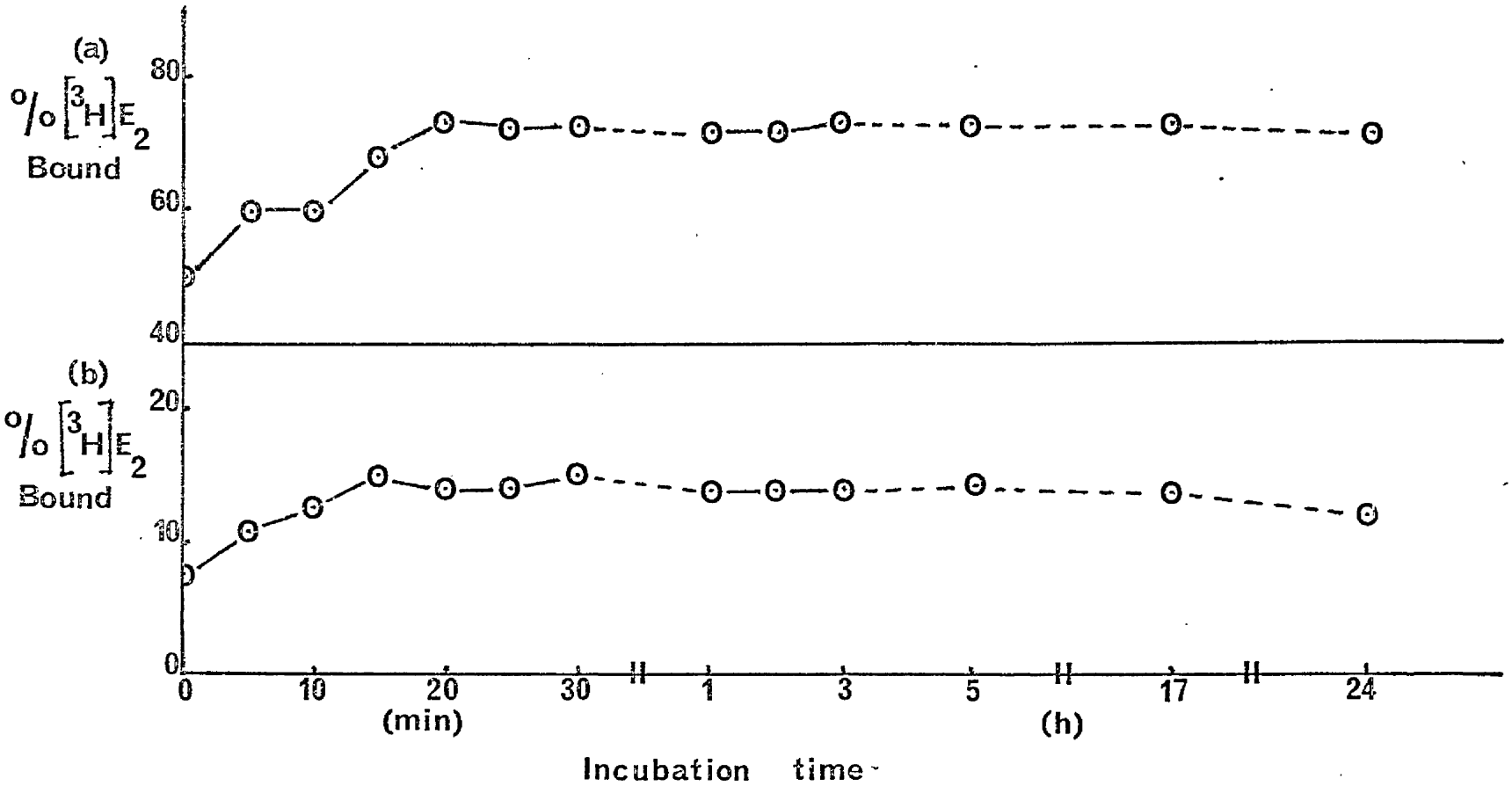
TABLE 3. 6. THE EFFECT OF CHARCOAL 'CONTACT TIME' ON THE APPARENT MAXIMUM BINDING OF $\left[^{3}H\right]$ OESTRADIOL TO ANTISERUM.

CHARCOAL 'CONTACT TIME' (min)	APPARENT MAXIMUM BINDING (%)
2	75
5	55
10	28

7. Incubation times and temperatures. In a preliminary experiment, incubation of standards with the antiserum solution and $\left[^{3}H\right]$ oestradiol, at $4^{o}C$, was carried out for 0, 5, 10, 15, 20, 25, 30 minutes and 1, 2, 3, 5, 17 and 24 hours. The percentage $\left[^{3}H\right]$ oestradiol bound when no 'cold' oestradiol was present and the percentage bound when 300 pg of 'cold' oestradiol were present, at various incubation times, are shown in Fig. 3. XII.

On the basis of these results, 20 minutes was chosen

FIG. 3. XII THE EFFECT OF INCUBATION TIME AT 4°C ON THE PERCENTAGE OF $[^3H]$ OESTRADIOL (E_2) BOUND WHEN a) NO 'COLD' OESTRADIOL WAS PRESENT AND b) 300 pg of 'COLD' OESTRADIOL WERE PRESENT.



as a suitable incubation time. Maximum binding and displacement of $\left[^{3}H\right]$ oestradiol (by 300 pg of 'cold' oestradiol) were achieved in this time. The 20 minutes incubation was most practical in the assay and more convenient than a 2 hour incubation (Wu and Lundy, 1971).

8. Measurement of the distribution of oestradiol between free and bound fractions. This measurement was made by determining the radioactivity in an aliquot of supernatant, obtained after charcoal precipitation and centrifugation, and comparing this with the total amount of $\left[^{3}H\right]$ oestradiol present as for the CPB assay (p. 44).

9. Comparison of unknown samples with a standard curve and calculation of results. A range of standard samples was prepared in duplicate (0 to 300 pg) from the stock (1 ng/ml) solution of oestradiol in methanol and the solvent evaporated. Unknown samples were determined by direct comparison of percentage $\left[^{3}H\right]$ oestradiol bound with the standard curve. Results were expressed in pg/ml of plasma. No recovery corrections were made.

10. Assessment of methodological interfering factors.

The problems of assessment of oestradiol methodological blanks were identical to those for progesterone and 17α-hydroxyprogesterone (p. 45). Water (0.2 ml) was used in the present study to determine the method blank. To minimise this blank it was particularly important to use freshly washed tubes and recently distilled ether. A bottle of ether which had been open for several days gave significantly high blank values. The method blank obtained was 3 ± 2 pg S.D. (n = 50).

11. Statistical assessment of the method.

a) Accuracy. Ten replicate samples each of 20 pg and 60 pg of authentic oestradiol were added to blanks and assayed. Accuracies of 93.5% (20 pg) and 108.9% (60 pg) were obtained.

b) Precision. The intra-assay coefficient of variation for 20 pg of oestradiol was ± 22.8% and for 60 pg ± 10.2%.

The inter-assay coefficient of variation was obtained

by assaying several samples from a quality control plasma pool with each assay. The results for these samples (n = 50) gave a value of ± 11.3% at the 60 pg per sample level.

c) Sensitivity. The lowest level of detection of oestradiol was estimated at 5 pg per sample (blank plus one S.D.).

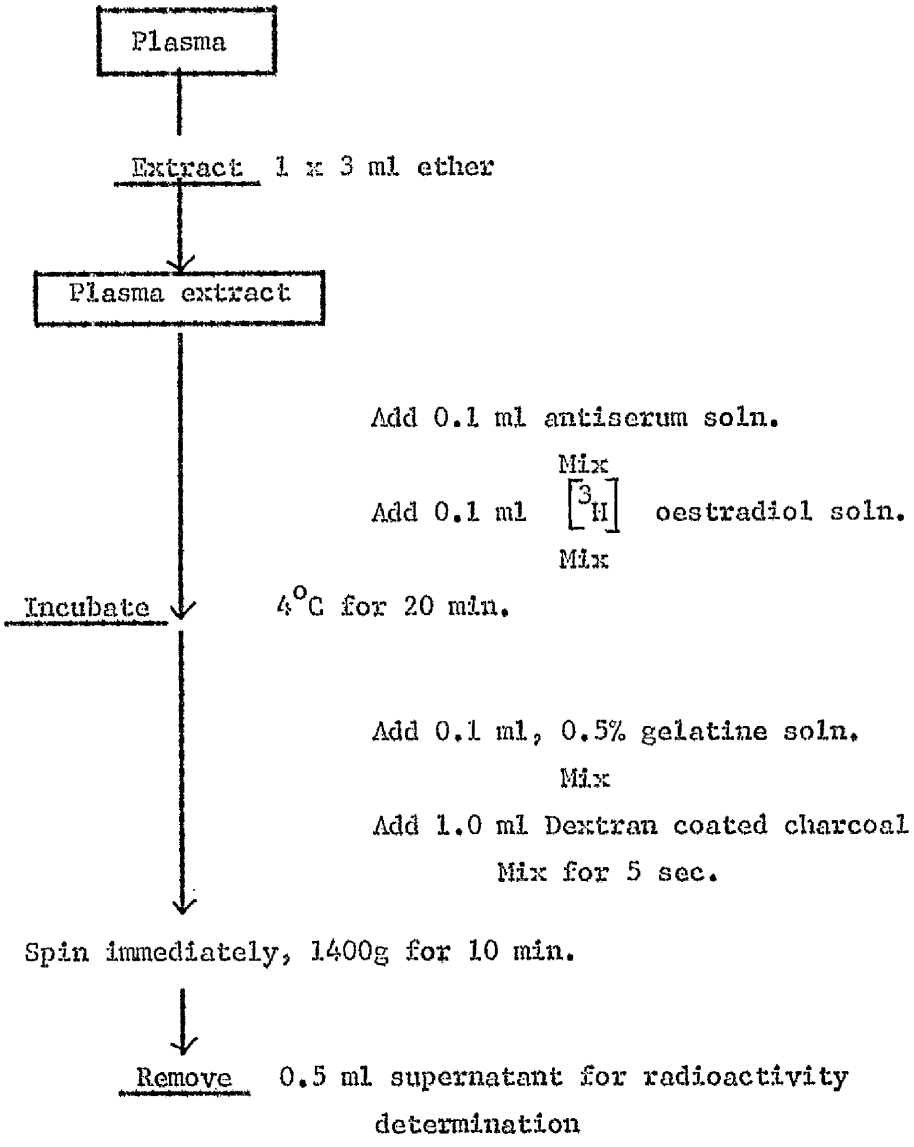
The method developed for the assay of oestradiol incorporating these criteria is summarised below:-

Assay of oestradiol by RIA.

Plasma (0.2 ml) was extracted once with 3 ml of ether by shaking for forty-five seconds, the tubes placed in crushed, solid CO_2 and the ether layer decanted into disposable assay tubes after freezing of the aqueous layer. The ether was evaporated and the samples subjected to RIA.

A range of standard samples was prepared from 0 - 300 pg, in duplicate, using the stock solution of oestradiol (1 ng/ml) in methanol and the methanol evaporated.

FIG. 3. XIII FLOW SHEET OF THE RADIOIMMUNOASSAY OF OESTRADIOL



0.1 ml of antiserum solution was added to all unknown samples and the standard samples and the contents of the tubes mixed gently. 0.1 ml of $\left[^{3}H\right]$ oestradiol (25,000 dpm) was added and the tubes' contents were again mixed gently. After incubation at $4^{o}C$ for twenty minutes, 0.1 ml of 0.5% gelatine solution was added to each tube and the contents mixed gently. 1 ml of dextran coated charcoal was then added to each tube, (except the 'no charcoal' tubes) the contents were mixed vigorously for five seconds and centrifuged immediately at 1400 g for ten minutes. 0.5 ml of the supernatant was removed for radioactivity determination.

Results were calculated as percentage $\left[^{3}H\right]$ oestradiol remaining bound after displacement by 'cold' oestradiol, using the results obtained for the 'no charcoal' tubes to calculate the total amount of $\left[^{3}H\right]$ oestradiol present. Unknown samples were compared with the standard curve. Fig. 3. XIII. is a flow sheet of the assay procedure.

CHAPTER 4

STEROID AND GONADOTROPHIN HORMONE PATTERNS THROUGHOUT MENSTRUAL CYCLES OF VOLUNTEER SUBJECTS

STEROID AND GONADOTROPHIN HORMONE PATTERNS THROUGHOUT MENSTRUAL CYCLES OF VOLUNTEER SUBJECTS

INTRODUCTION

Plasma levels of steroid and gonadotrophin hormones throughout menstrual cycles from apparently normal volunteers were established in this study for several reasons. Firstly, it was important to establish the sequence of hormonal events in the normal cycle. Secondly, confirmation of the reliability of the assay methods was obtained by comparison of the hormone levels found in the normal cycle with those reported by other workers. Finally normal ranges for hormone levels were established for comparison with cycles from infertile subjects. This comparison may be important in defining the cause of infertility since small differences between normal and infertile cycles in hormone levels or patterns may be significant.

Whilst mean levels of hormones throughout normal cycles are not only useful for establishing a normal range but necessary for comparison with 'infertile' cycles, much important information may be lost if cycles are not studied individually. Examination of individual cycles

shows the pattern of each hormone in the same cycle and allows a more direct study of possible inter-relationships between hormones to be made. In this thesis therefore, the hormone levels are presented not only as the mean of all the cycles for each hormone, but also as the hormone patterns in each individual cycle.

Problems arise in defining a 'normal' cycle. Certain broad criteria must be laid down when selecting subjects and cycles for inclusion in a normal series. Ideally, perhaps, the subjects should be of proven fertility but such volunteers, who are not practising contraception, are difficult to find. Nineteen cycles from fourteen women have been studied. These women were apparently normal, aged 18 to 26 years, unmarried and nulliparous. The criteria used initially in selecting normal subjects were :-

1) A history of regular menstrual cycles of between 27 and 32 days.

2) No history of general medical or gynaecological abnormality.

3) No history of oral contraception.

'Normal' cycles were defined, for the purposes of this study, as :-

1) Cycles having a luteal phase of at least 13 days (Rock, 1949; Vande Wiele and Turksoy, 1965) measured from the day of the maximum LH value until the onset of the following menses.

2) Cycles being obtained from subjects having no known abnormalities or special peculiarities.

Using this definition only 8 of the 19 cycles studied could be strictly classified as 'normal'.

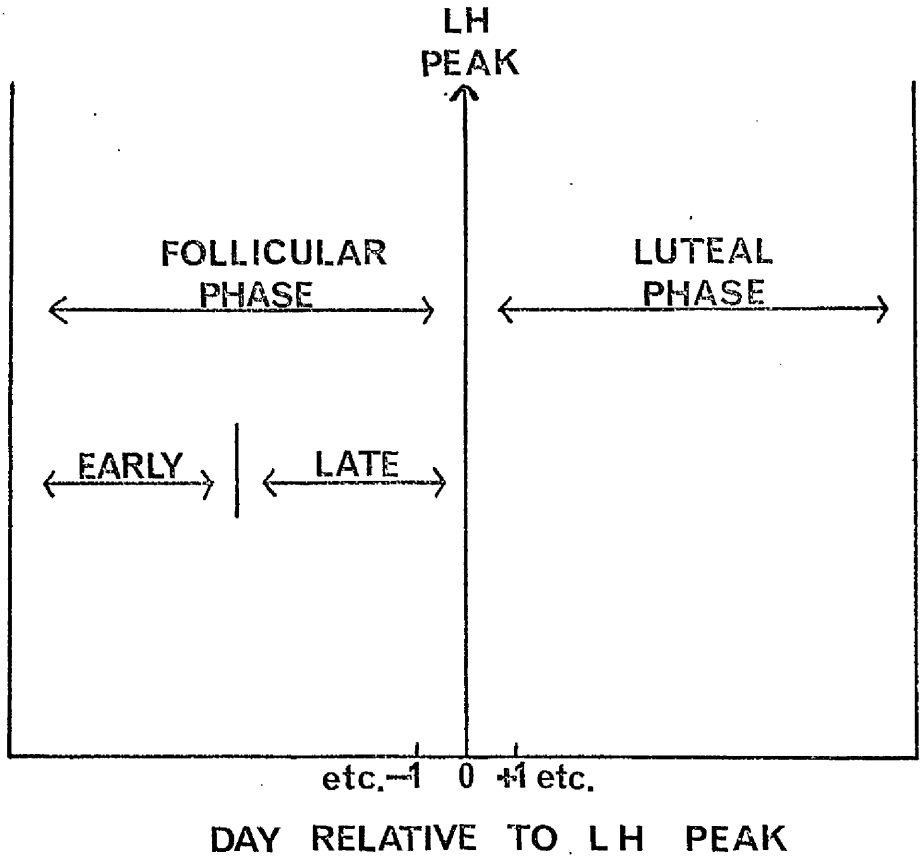
In presenting the data, the onset of menses was used as a reference point to mark the beginning and end of sample collection periods. The LH mid-cycle peak, which is traditionally considered to be the "most accurate indicator of the phases of the menstrual cycle" (Ross et al., 1970) has been used as a marker on which to base data. This was especially useful when calculating means and defining intervals in order to eliminate the effects of varying cycle length and time of ovulation. The scheme used for presenting

cycles in this, and the following chapters is shown in Fig. 4. I. The day of the LH mid-cycle peak was designated as day 0.

Daily blood samples were collected from an arm vein at 09.00 hours each morning throughout 19 menstrual cycles, into lithium heparin tubes. The subjects were sedentary during this procedure. The plasma was separated immediately by centrifuging for 10 minutes in a bench centrifuge. Aliquots were removed for LH and FSH determinations and the remainder was frozen at -20°C. until assays of steroid hormones were carried out.

Progesterone and 17α-hydroxyprogesterone were assayed in the same 1 ml sample of plasma by the CPB method and oestradiol in 0.2 ml of plasma by the RIA method which have been described in Chapter 3 and summarised in Fig. 3. VI and VII and Fig. 3. XIII respectively. LH and in some cycles, FSH, were measured in other laboratories as previously described (p. 28). Aliquots of the same sample were used for all estimations and samples from an entire cycle were determined in a single assay. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phases of the cycles were measured.

FIG. 4. I THE SCHEME USED FOR PRESENTING MENSTRUAL CYCLE DATA. THE DAY OF THE LH MID-CYCLE PEAK IS DESIGNATED AS DAY 0.



The areas under the curves were defined as the total amount of plasma steroid measured from day +1 to the day on which the next menstrual bleeding began inclusive. In some cycles, basal body temperature (BBT) was recorded, orally, by the subjects themselves before rising each morning throughout the cycle studied. No restrictions were placed upon physical activities or diet. Neither endometrial nor ovarian biopsy was performed in these subjects. Brief details of the subjects studied are presented in Table 4. 1.

RESULTS

The results are presented in two parts. The cycles which are considered normal are presented in Part I and the remaining 11 cycles in Part II, where the reasons for their separate presentation will be discussed.

PART I

HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE

A. MEAN HORMONE LEVELS

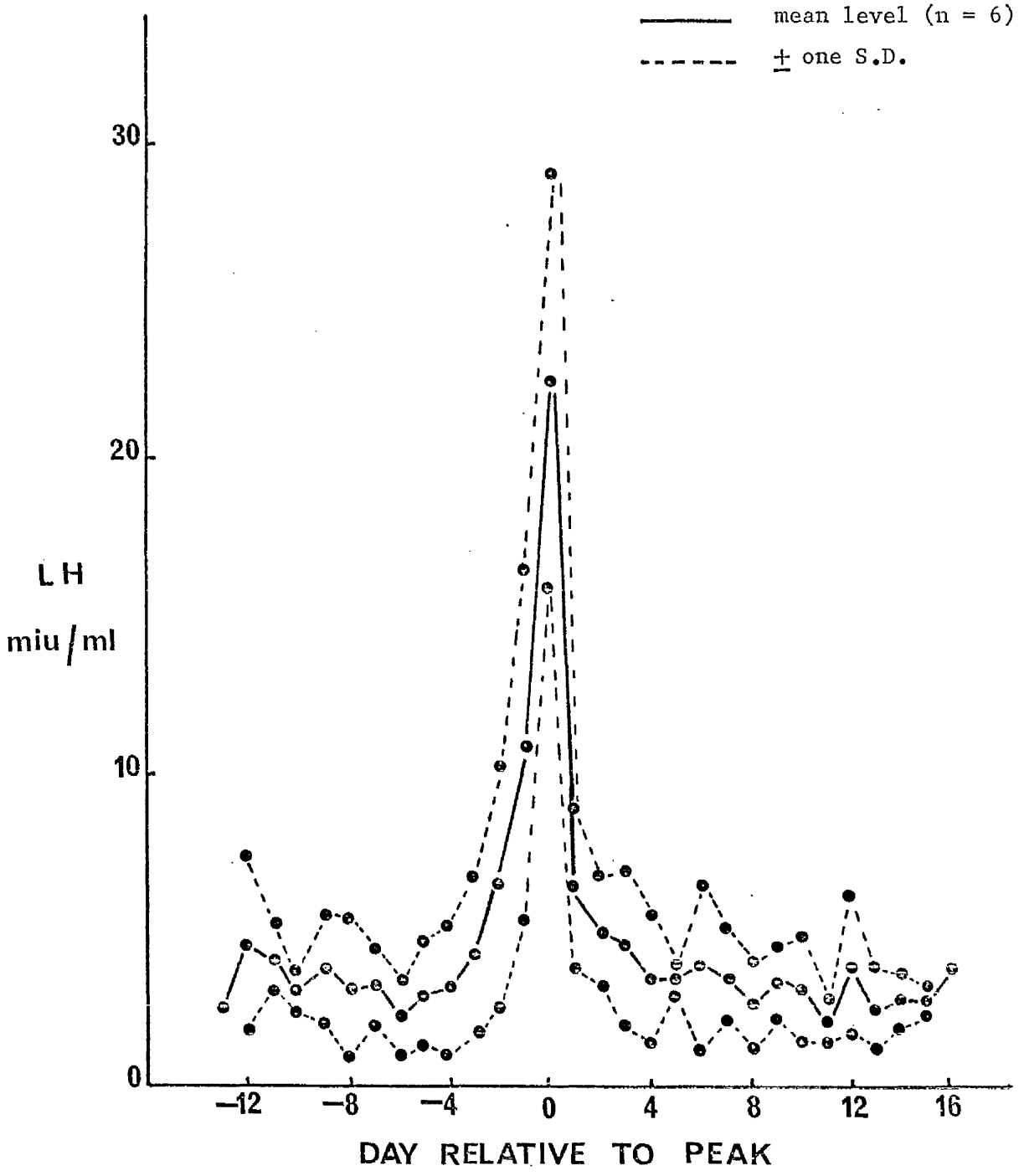
Fig. 4. II shows the mean LH pattern $\pm$ S.D. (n = 6).

TABLE 4. 1. DETAILS OF THE APPARENTLY NORMAL VOLUNTEER SUBJECTS STUDIED IN CHAPTER 4.

NAME	AGE	CYCLE LENGTH	LUTEAL PHASE LENGTH
KB	22		
Cycle 1		31	13
Cycle 2		32	10
PH	23		
Cycle 1		24	14
Cycle 2		27	14
Cycle 3		26	14
MF	25	31	14
SR	25	29	12
MA	20	28	11
JG	26		
Cycle 1		30	9
Cycle 2		30	14
KK	25	29	17
BW	20		
Cycle 1		31	12
Cycle 2		30	10
CW	21	27	12
SH	22	28	15 *
EP	20	27	13
HG	22	32	14
DB	24	29	16
MMc	22	28	15

* Calculated from 1st day of LH rise

FIG. 4. II THE MEAN LEVELS OF LH ± S.D. (n = 6) THROUGHOUT THE NORMAL MENSTRUAL CYCLE. DAYS ARE NUMBERED RELATIVE TO THE LH PEAK IN THIS AND SUBSEQUENT DIAGRAMS



Levels began at 4 m.i.u./ml on day -12 and showed a downward trend to 2 m.i.u./ml on day -6. From then, levels began to rise steadily until day -2 and then sharply to a mid-cycle peak (day 0) of 22 ± 7 (S.D.) m.i.u./ml. Levels then dropped steeply until day +2 and more gradually until day +5 whence they remained steady until the end of the cycle. Peak values ranged from 15 m.i.u./ml to 32 m.i.u/ml.

Fig. 4. III shows the mean FSH pattern ± S.D. (n = 5). Levels began at around 18 m.i.u./ml on day -13. They then dropped gradually to 6 m.i.u./ml by day -5. The FSH mid-cycle peak was not as well defined as the LH peak and peak levels extended over 3 days from day -1 to day +1. Levels then dropped gradually reaching a second nadir on day +10. A sharp rise occurred, on day +14, to a level comparable to that at the beginning of the cycle. The standard deviation was very large throughout. Mid-cycle peak levels (day -1) ranged from 10 - 21 m.i.u./ml.

Fig. 4. IV shows the mean progesterone pattern ± S.D. (n = 8). The mean follicular phase level was around 1 ng/ml and remained steady throughout this phase. Levels rose significantly from day +1 onwards and continued to rise consistently to a peak value of 8 ng/ml on day +5.

FIG. 4. III THE MEAN LEVELS OF FSH ± S.D. (n = 5) THROUGHOUT THE NORMAL MENSTRUAL CYCLE

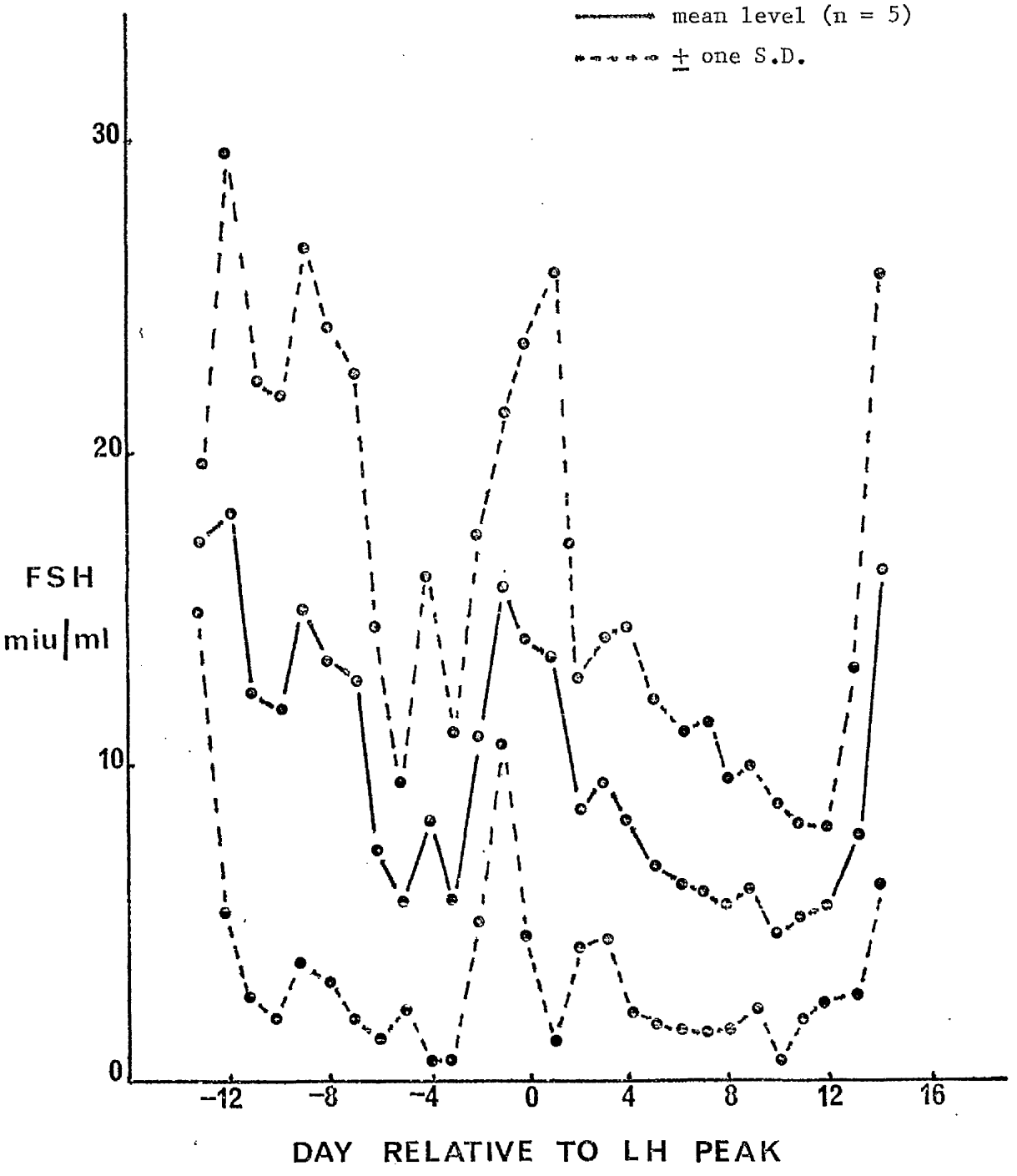
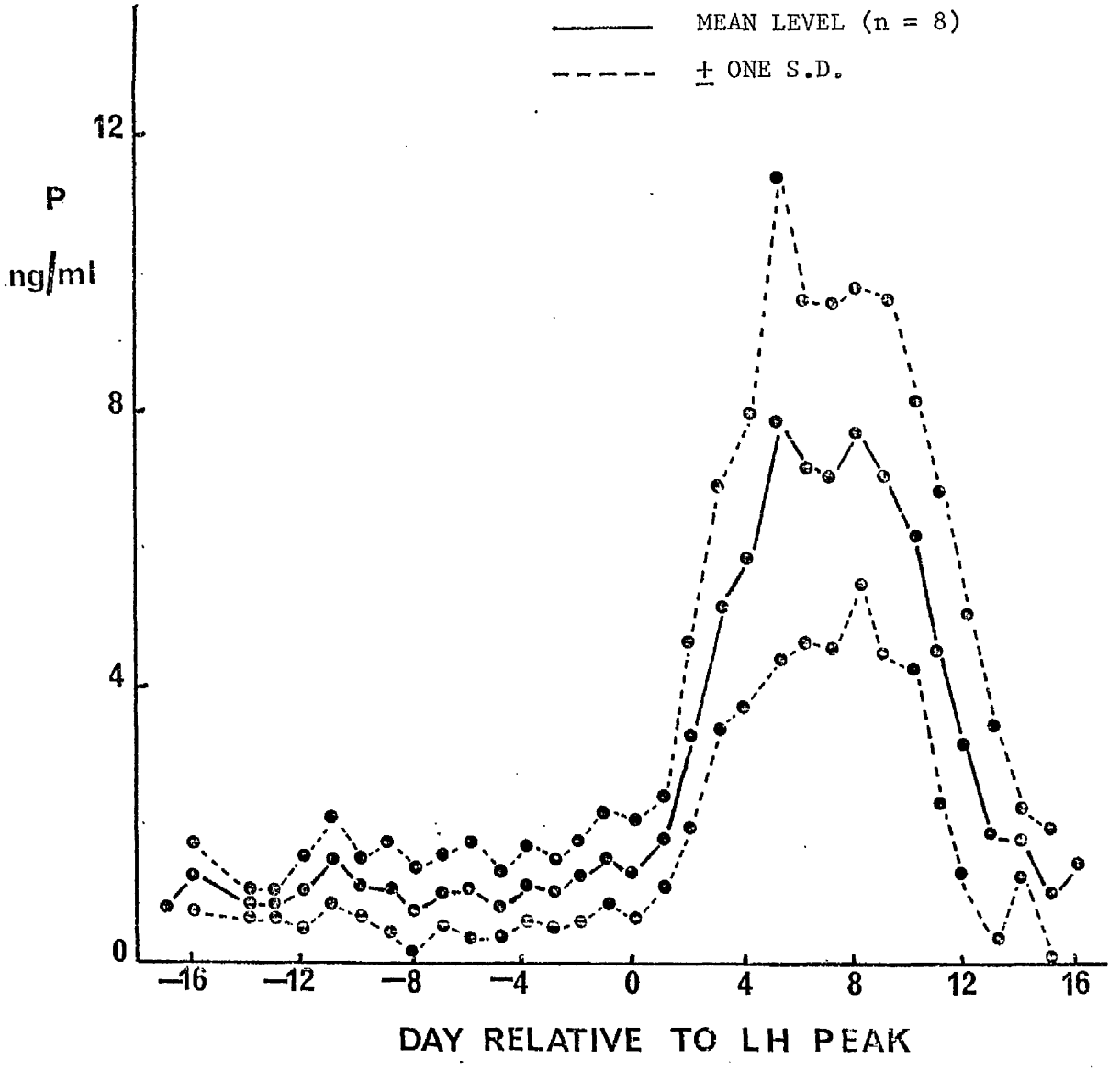


FIG. 4. IV THE MEAN LEVEL OF PROGESTERONE (P) ± S.D. (n = 8) THROUGHOUT THE NORMAL MENSTRUAL CYCLE.



Levels remained high until day +8 when they dropped, reaching 1 ng/ml by day +15. The standard deviation was large, thus, for example luteal peak levels on day +5 ranged from 4.5 to 11.5 ng/ml and early follicular phase levels ranged from around 0.5 to 1.5 ng/ml.

Fig. 4. V. shows the mean 17α-hydroxyprogesterone pattern ± S.D. (n = 8). Mean levels began at 0.8 ng/ml and showed a downward trend to 0.4 ng/ml on day -11. They then rose but remained below 0.8 ng/ml until day -1 when a significant increase occurred culminating in a discrete peak with a mean value of 1.5 ng/ml on day 0. A drop of approximately 15 - 20% was observed on day +1, followed by a steady rise to a luteal phase plateau of around 2.4 ng/ml between days 5 and 8. Levels dropped consistently to a mean value of 0.6 ng/ml on day +14 but rose to around 1.0 ng/ml by the end of the cycle. The standard deviation was again large in both follicular and luteal phases.

Fig. 4. VI shows the mean oestradiol-17β pattern ± S.D. (n = 8). Mean levels began at just below 100 pg/ml and fluctuated between 100 and 150 pg/ml until day -5 when a steady increase began culminating in a peak of 320 pg/ml which occurred on the day before the LH peak (day -1).

FIG. 4. V THE MEAN LEVEL OF 17α-HYDROXYPROGESTERONE (17 OHP) $\pm$ S.D. (n = 8) THROUGHOUT THE NORMAL MENSTRUAL CYCLE.

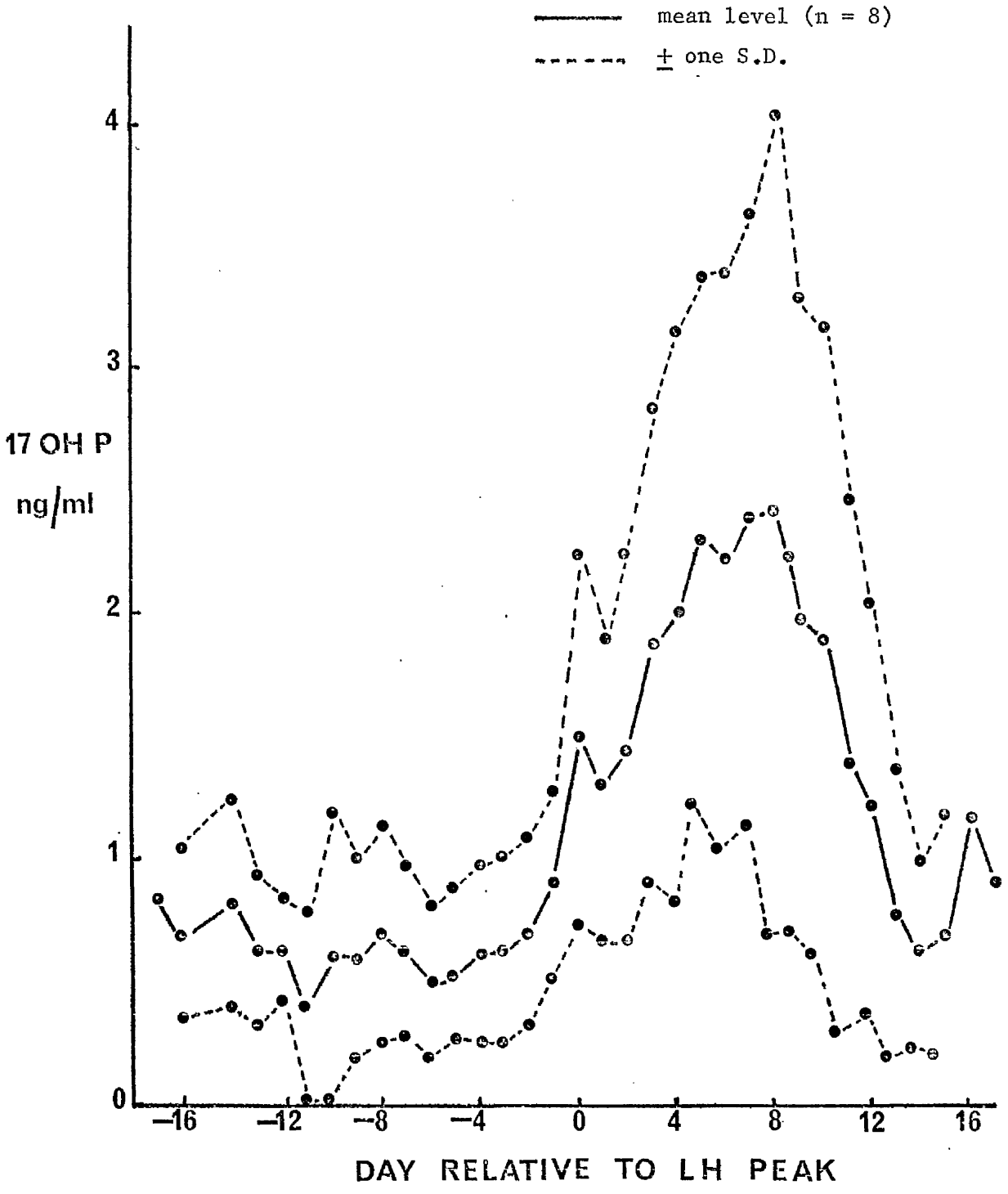
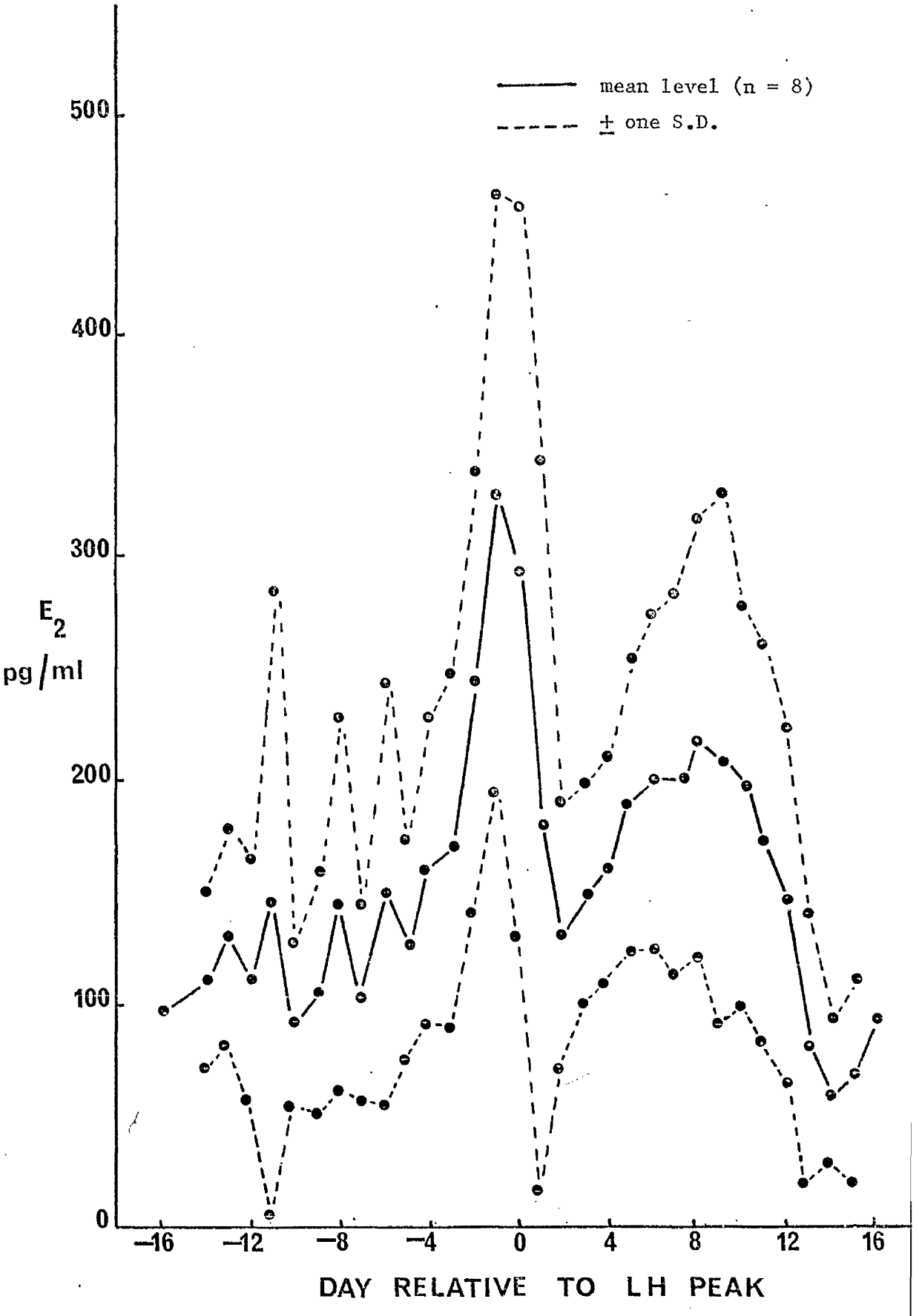


FIG. 4. VI THE MEAN LEVEL OF OESTRADIOL (E_2) ± S.D. (n = 8) THROUGHOUT THE NORMAL MENSTRUAL CYCLE.



Levels dropped steeply to about 60% of the peak value and then rose in the luteal phase, reaching a plateau between days +5 and +10. This luteal peak (220 pg/ml) was always of less magnitude than the mid-cycle one. Levels dropped steadily after day +10 reaching 70 pg/ml on day +15. The standard deviation was again large in both the follicular and luteal phases.

B. HORMONE LEVELS IN INDIVIDUAL CYCLES

Hormone patterns in the individual normal cycles which comprise the mean cycle are presented in diagramatic form. Outstanding features of these cycles which were less well defined or absent in the mean cycle are described in the text.

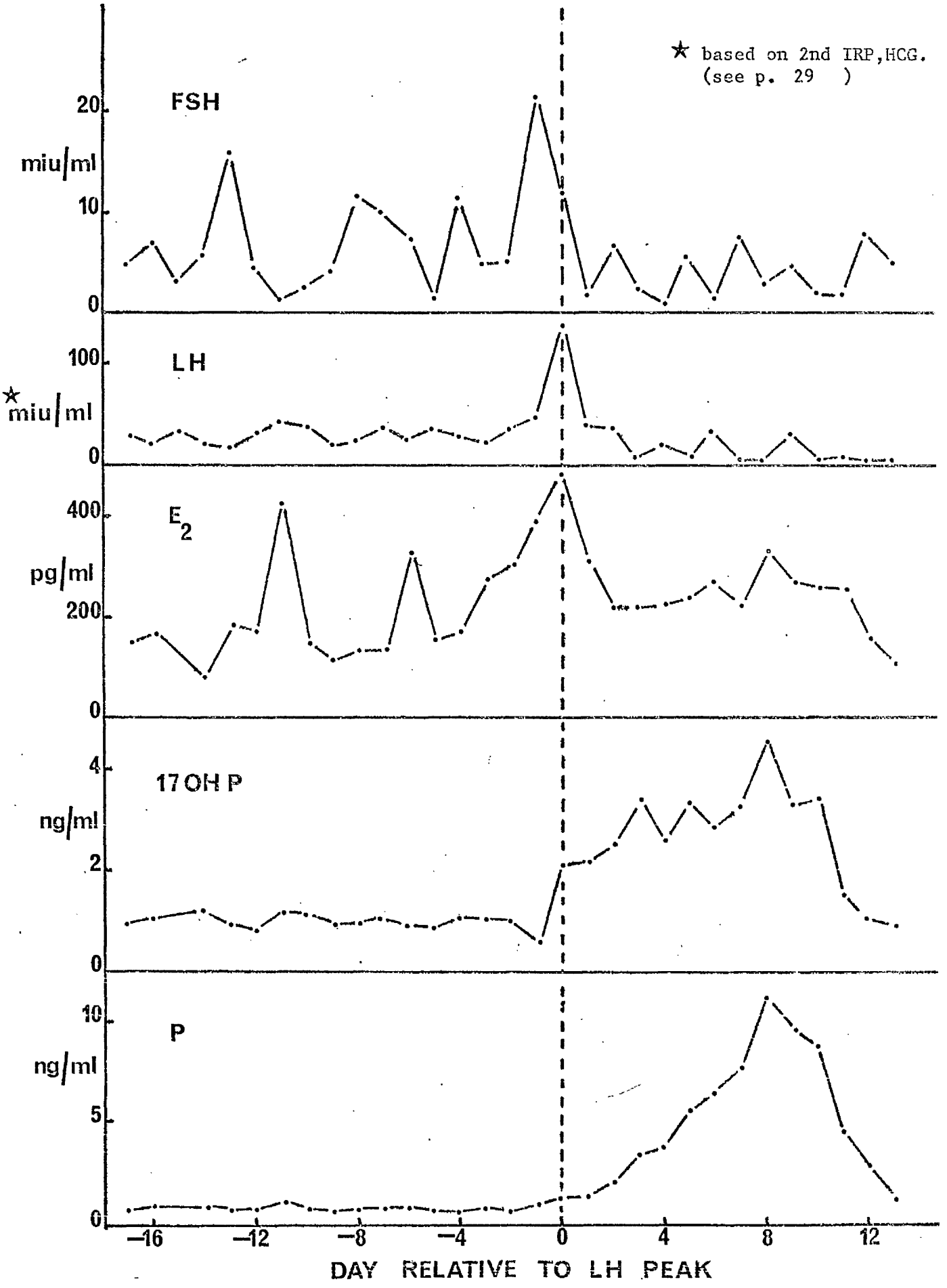
Subject KB (Cycle 1)

This subject was the only one included in the study who, in a later cycle, became pregnant and was therefore the only one of proven fertility. She was admitted for therapeutic abortion at 12 week gestation and blood samples were collected throughout a cycle (cycle 2) some months after this, which will be presented in Part II of this chapter.

The results for Cycle 1 are shown in Fig. 4. VII.

FIG. 4.VII STEROID AND GONADOTROPHIN HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE.

SUBJECT K.B., Cycle 1.

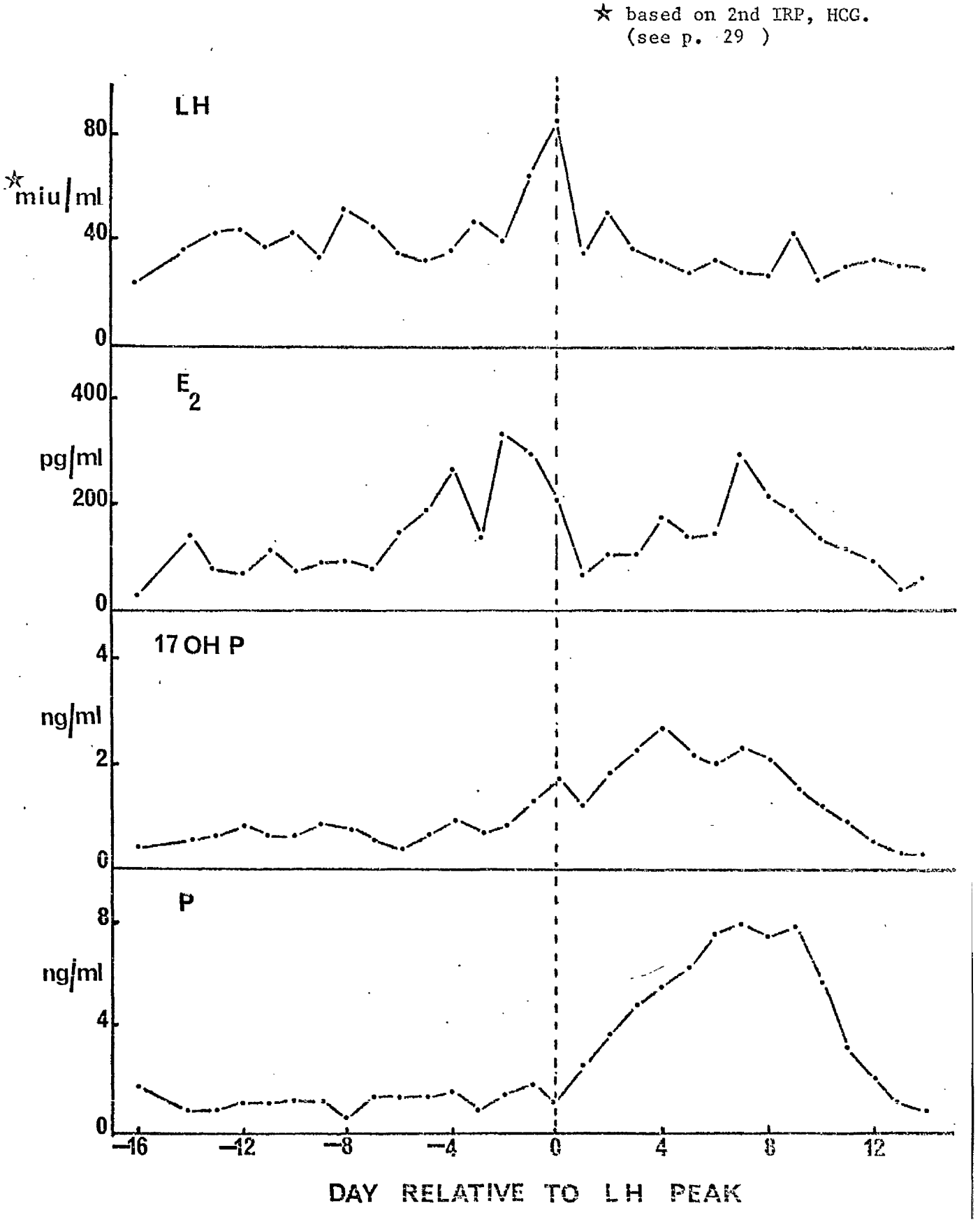


A possible relationship existed between FSH and oestradiol in the follicular phase of the cycle. An FSH peak on day -13 preceded an oestradiol peak on day -11 and a second FSH peak on day -8 preceded a second oestradiol peak on day -6. A third FSH peak on day -4 preceded a rise in oestradiol on day -3 which culminated in the mid-cycle peak of oestradiol, occurring on the same day as the LH peak. A mid-cycle FSH peak occurred on the day before the LH and oestradiol peaks. No discrete 17α-hydroxyprogesterone peak was present but a rise occurred on day 0 and was sustained into the luteal phase. All three steroid hormones reached luteal phase peaks on day +8 and their patterns were similar in the late luteal phase; the three hormones dropped by 50% of their value between days +10 and day +11. The BBT curve was biphasic, the temperature rise being apparent on day +2. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase of the cycle were 70.2 ng, 34.6 ng and 3150 pg respectively.

Subject MF

The results for this subject are shown in Fig. 4. VIII.

FIG. 4. VIII STEROID AND GONADOTROPHIN HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE.
SUBJECT M.F.

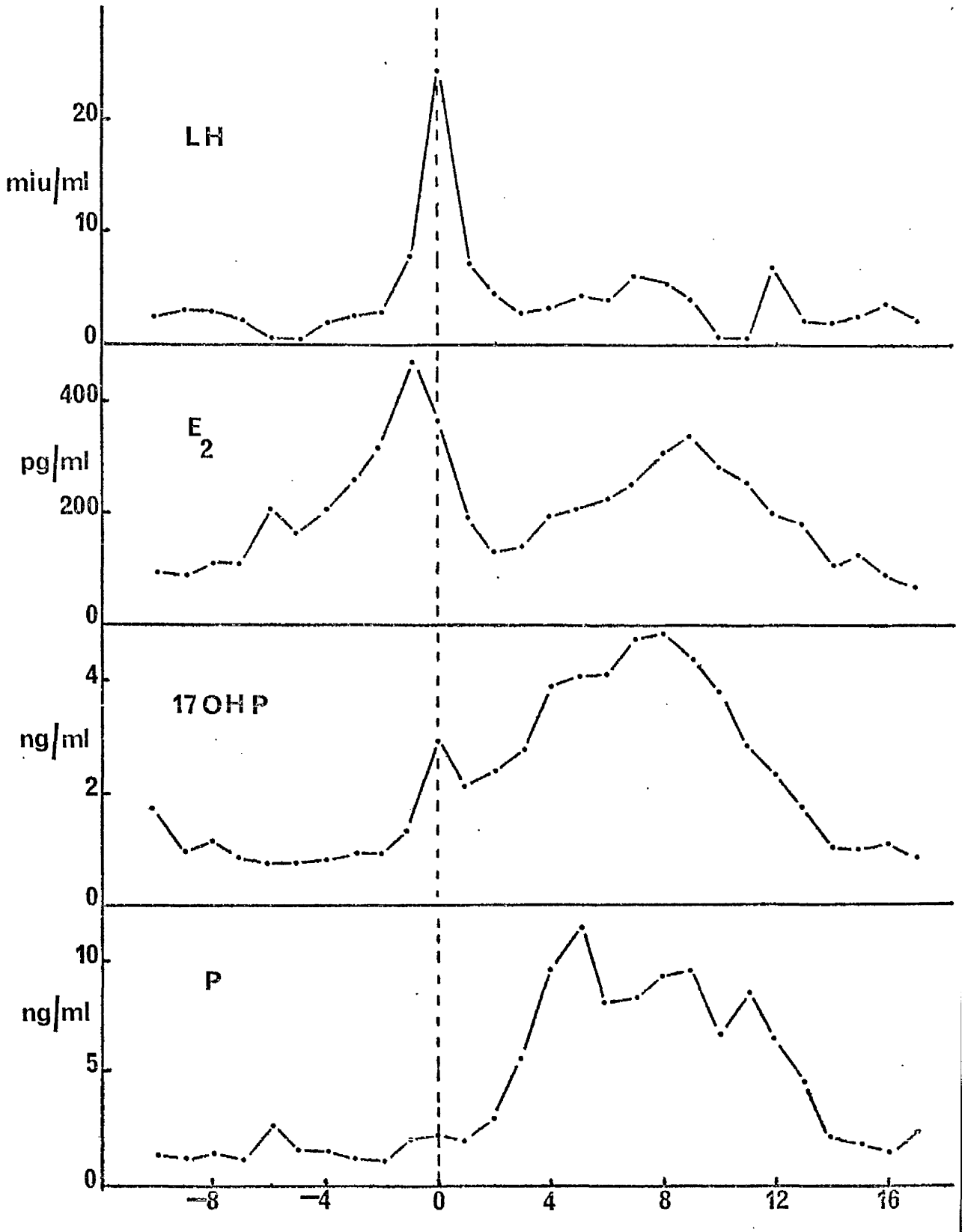


Two major oestradiol peaks occurred in the late follicular phase of the cycle, one on day -4 and the second on day -2, two days before the LH mid-cycle peak. However, oestradiol remained at peak levels until day -1 when the first rise in LH occurred. Oestradiol levels dropped on day +1 by 75% of their mid-cycle peak value and rose in the luteal phase to a peak on day +7 which was higher in relation to the mid-cycle peak than in the mean cycle. A discrete 17α-hydroxyprogesterone peak occurred on day 0. The BBT curve was bi-phasic and showed a significant increase in temperature on day +1. The areas under the progesterone, 17α-hydroxy-progesterone and oestradiol curves in the luteal phase of this cycle were 68.2 ng, 24.7 ng and 1865 pg respectively.

Subject KK

The results for subject KK are shown in Fig. 4. IX. This cycle had a short follicular phase (10 days) and a long luteal phase (17 days). However, it followed the mean pattern in most respects. The mid-cycle oestradiol peak occurred on the day before the LH peak and a discrete 17α-hydroxyprogesterone peak occurred on the day of the LH peak. Progesterone showed a small but

FIG. 4. IX STEROID AND GONADOTROPHIN HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE.
SUBJECT K.K.

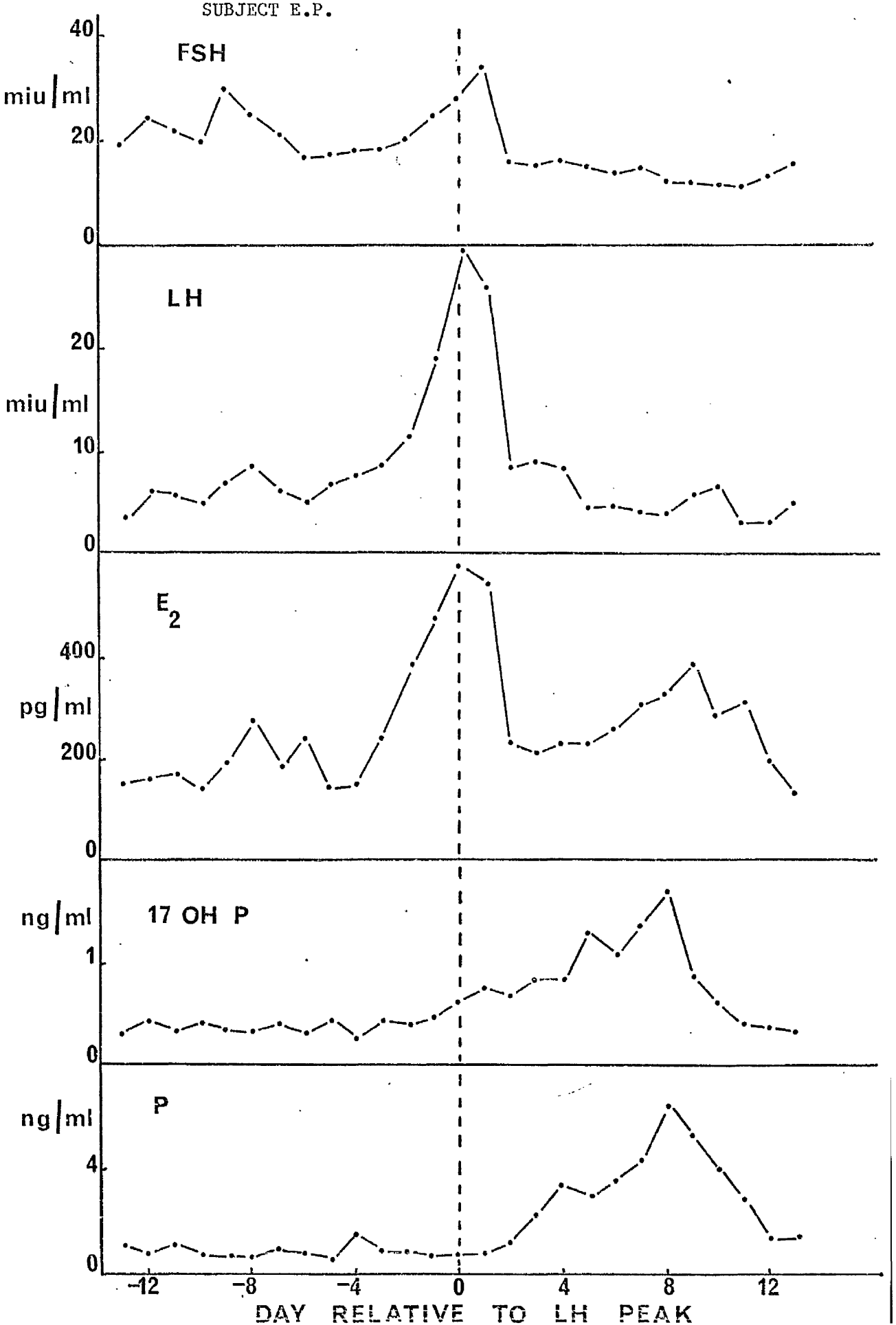


significant rise from day -2, coincident with the LH rise and before the LH peak, which was sustained into the luteal phase. The BBT curve was bi-phasic, showing a significant increase in temperature on day +3. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase of the cycle were 102.8 ng, 48.5 ng and 3270 pg respectively.

Subject EP

The results are shown in Fig. 4. X. A peak of FSH occurred on day -9 and an oestradiol peak on day -8. The mid-cycle oestradiol peak, the value of which was above one S.D. of the mean cycle, occurred on the same day as the LH peak and the mid-cycle FSH peak occurred on day +1. The LH peak value was also above one S.D. of the mean cycle. There was no discrete mid-cycle 17α-hydroxyprogesterone peak but levels rose from day -1 to day +2, and the rise continued into the luteal phase. Both progesterone and 17α-hydroxyprogesterone levels were low in the luteal phase and showed a similar pattern, both reaching peaks on day +8. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase were 39.3 ng, 11.1 ng and 3000 pg respectively.

FIG. 4. X STEROID AND GONADOTROPHIN HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE.
SUBJECT E.P.



Subject MMc

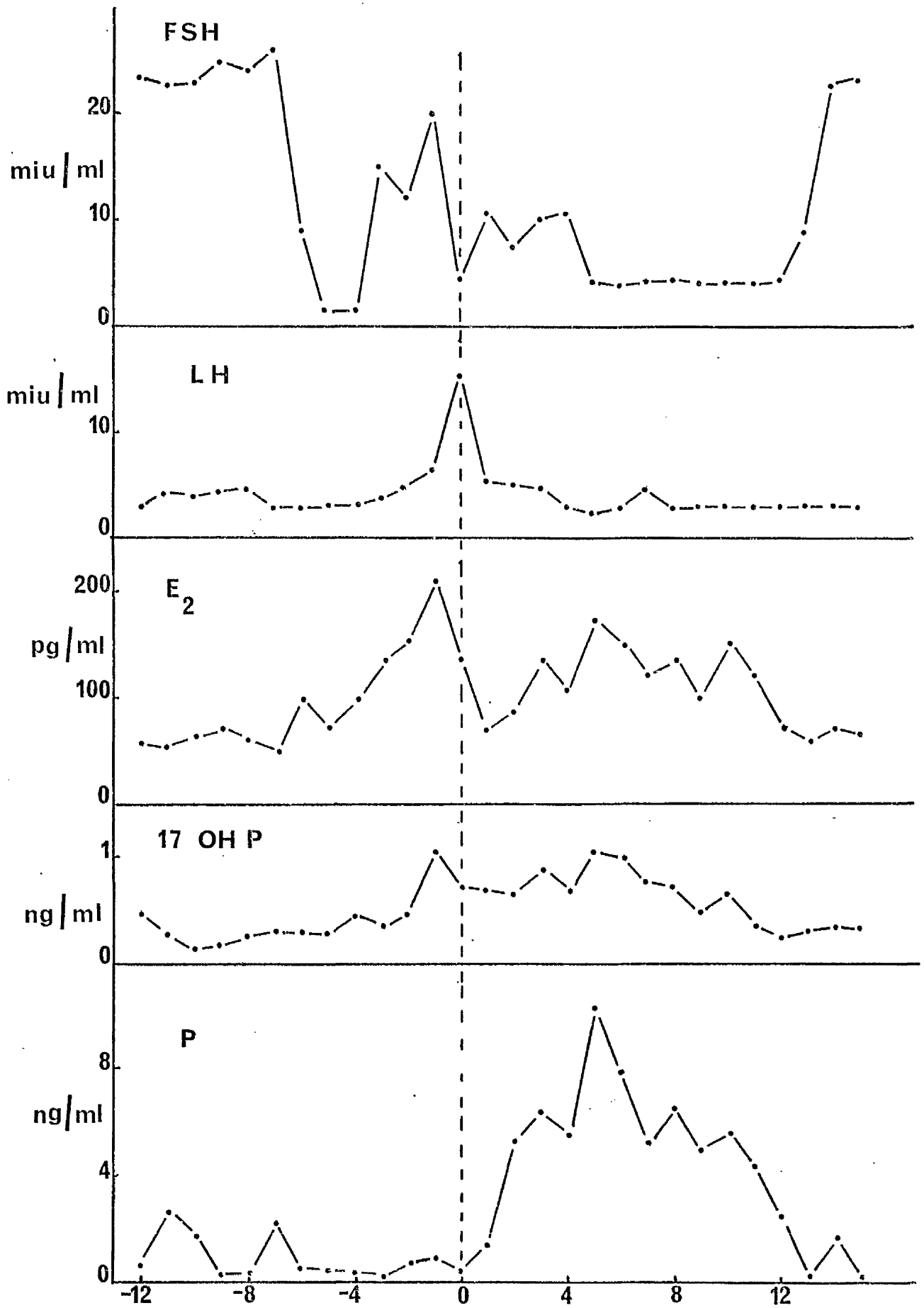
The results are shown in Fig. 4. XI. FSH levels were consistently high from days -12 to -7 when they dropped dramatically. The first rise of oestradiol was concomitant with this drop. Mid-cycle peaks of oestradiol and FSH occurred on day -1. 17α-hydroxy-progesterone reached a small peak on day -1. Patterns of steroid hormones were similar in the luteal phase, although progesterone levels were high, whilst 17α-hydroxyprogesterone and oestradiol levels were low. FSH rose at the end of the cycle on day +13, when the steroid hormones had returned to base-line levels. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase were 68.4 ng, 8.9 ng and 1565 pg respectively.

Subject PH

This subject gave daily blood samples throughout three cycles. Some months after the first cycle (Cycle 1) she developed amenorrhoea, following a period of dieting associated with taking 2 x 10 mg 'Ponderax' tablets per day. Daily samples were collected during part of this period, presumably of anorexia nervosa, which

FIG. 4. XI STEROID AND GONADOTROPHIN HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE.

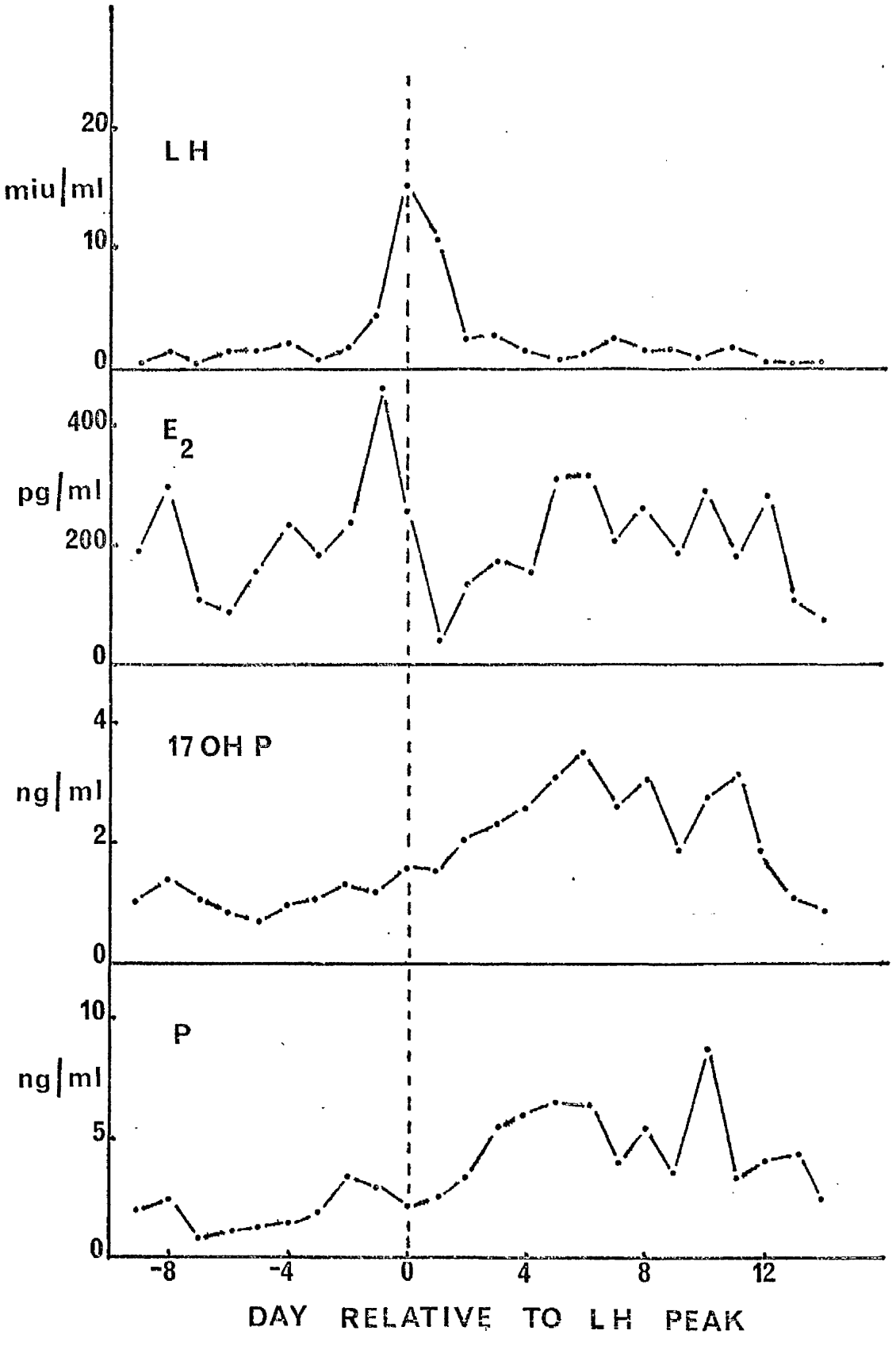
SUBJECT M.Mc



lasted approximately four months, and the results will be presented in the second part of this chapter. Six months after the return of normal menstruation she gave blood for two consecutive cycles (Cycles 2 and 3).

Cycle 1 - is shown in Fig. 4. XII. This was a short cycle (24 days) but nevertheless had a 14 day luteal phase. Oestradiol levels were high at the beginning of the cycle and a peak occurred on day -8 which was not accompanied by an increase in LH. FSH results were not available for this cycle. The mid-cycle oestradiol peak fell on the day before the LH peak. Oestradiol levels dropped by about 90% of their peak value on day +1 before the luteal phase rise began. There was no discrete mid-cycle 17α-hydroxyprogesterone peak, but a gradual rise began on day -5 and was continued into the luteal phase. There was a rise in progesterone on day -2, but a drop occurred on day 0 before the luteal phase rise began. The luteal phase patterns of the three steroid hormones were similar. The BBT curve was bi-phasic. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol cruves were 56.6 ng, 33.1 ng and 2830 pg respectively.

FIG. 4. XII STEROID AND GONADOTROPHIN HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE.
SUBJECT P.H., Cycle 1.



Cycle 2 - is shown in Fig. 4. XIII. This cycle was longer than the previous one, being 27 days but also with a 14 day luteal phase. Mid-cycle peak levels of oestradiol and FSH occurred on day -1 and FSH remained at its peak level until 4 days after the LH peak. The luteal phase levels of oestradiol were higher in relation to mid-cycle levels than in the mean cycle and they reached luteal peak levels on day +5. A discrete 17α-hydroxyprogesterone peak occurred on the day of the LH peak. 17α-hydroxyprogesterone and oestradiol patterns were similar in the luteal phase. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase were 69.6 ng, 19.5 ng and 1220 pg respectively.

Cycle 3 - is shown in Fig. 4. XIV. This cycle was similar in length to the previous one and again longer than Cycle 1, being of 26 days duration with a luteal phase of 14 days. It was interesting to note that in three cycles from the same subject, the luteal phase was constant in length whilst the follicular phase varied. A peak of oestradiol occurred on day -8

FIG. 4. XIII STEROID AND GONADOTROPHIN HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE.
SUBJECT P.H., Cycle 2.

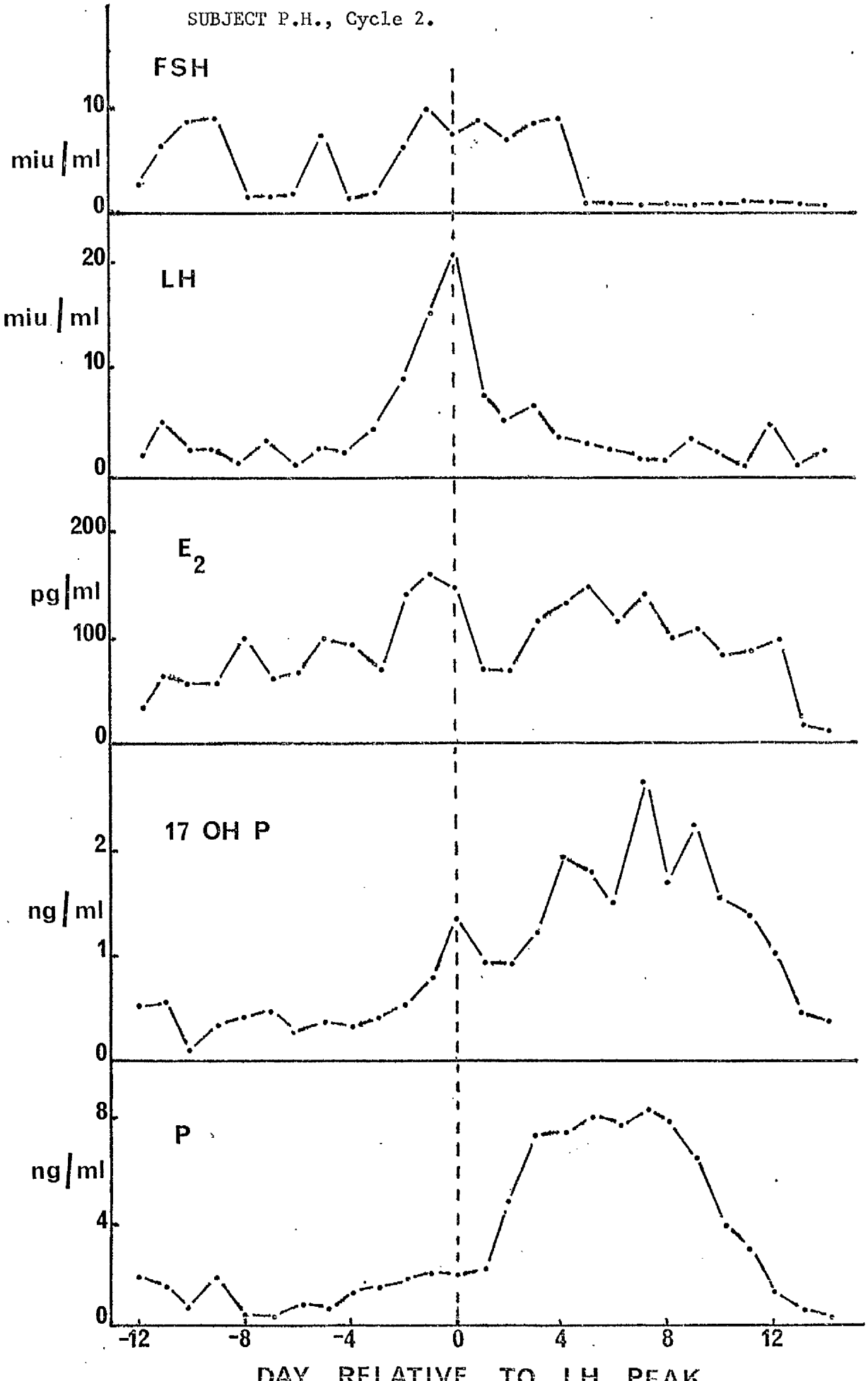
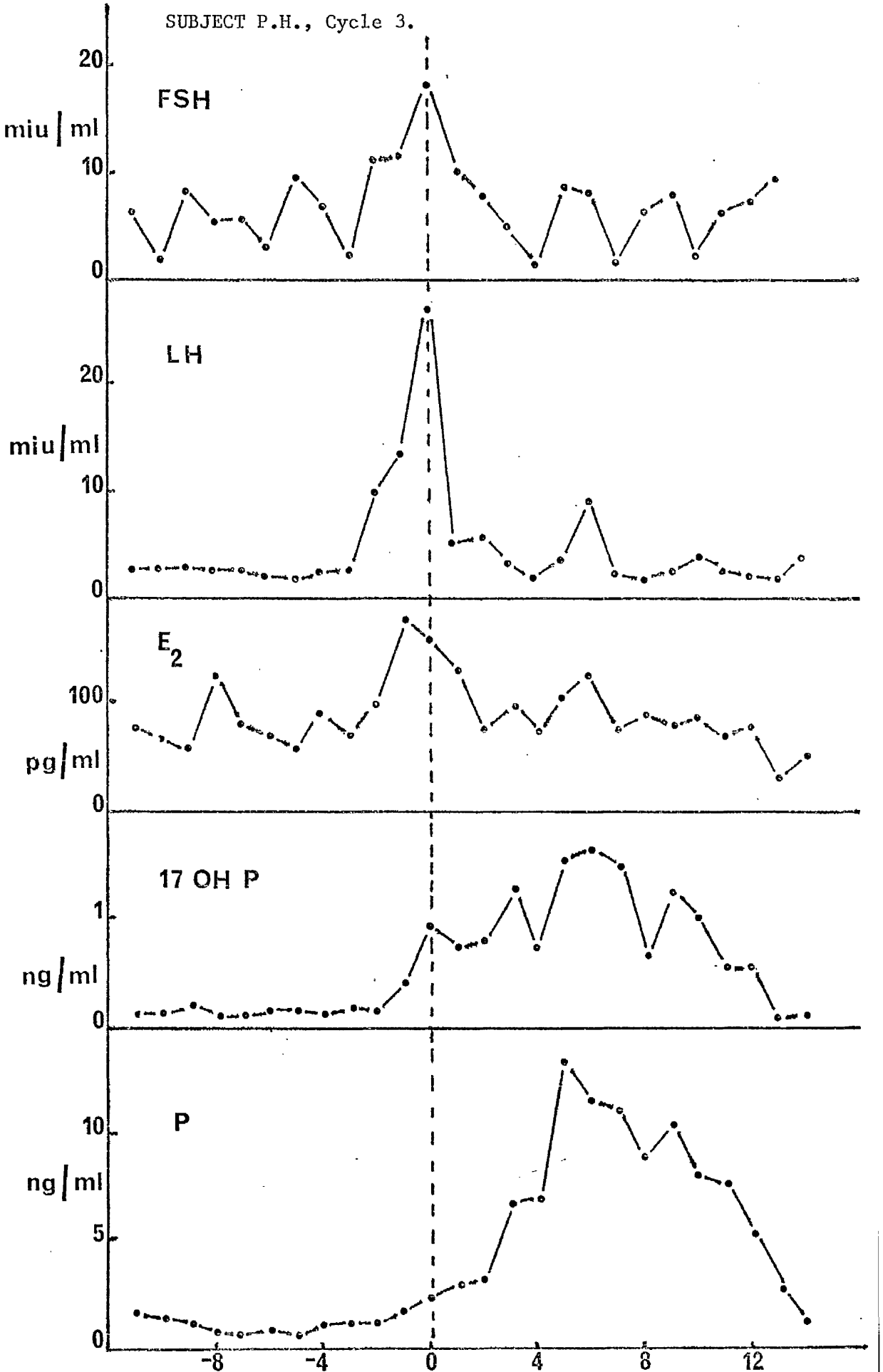


FIG. 4. XIV STEROID AND GONADOTROPHIN HORMONE PATTERNS IN THE NORMAL MENSTRUAL CYCLE.
SUBJECT P.H., Cycle 3.



though this was not associated with a change in gonadotrophin levels. The mid-cycle oestradiol peak occurred on day -1 and the mid-cycle FSH peak on day 0. A rise in 17α-hydroxyprogesterone occurred on day 0 and continued into the luteal phase. The patterns of the steroid hormones were similar in the luteal phase; however, progesterone levels were high whilst 17α-hydroxyprogesterone and oestradiol levels were lower than the mean. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase were 103.1 ng, 12.7 ng and 1200 pg respectively.

C. SUMMARY OF RESULTS IN NORMAL MENSTRUAL CYCLES

Certain major features were common to apparently ovulatory cycles. Other features were less well defined and differed greatly in individual cycles.

A peak of oestradiol secretion always either preceded by one or two days or was coincident with the mid-cycle peak of LH. In either case, levels of oestradiol were always beginning to rise at least one day before LH began to rise, and LH always rose before oestradiol levels began to decline. There appeared to be no correlation between peak heights of

oestradiol and peak heights of LH. For example in subject PH (Cycle 1), Fig. 4. XII, the LH peak height was low and was preceded by a high level of oestradiol; in subject EP (Fig. 4. X) both LH and oestradiol levels were high; in subject PH (Cycle 2) the LH level was high but was preceded by a low peak of oestradiol. The peak height of LH did not appear to be associated with the 'quality' of luteinization as reflected by progesterone secretion; in subject EP (Fig. 4. X), LH levels were high but luteal progesterone levels low, whilst a very low LH peak, in subject MMc (Fig. 4. XI) was followed by high luteal progesterone levels.

Mean FSH levels were high in the early follicular phase. The mid-cycle peak of FSH was not as well defined as the LH peak. In individual subjects there appeared to be some correlation between FSH secretion and oestradiol secretion, as in subject KB (Fig. 4. VII). These peaks of oestradiol were not associated with peaks of 17α-hydroxyprogesterone.

A discrete mid-cycle peak of 17α-hydroxyprogesterone was not always apparent but in all cases levels had risen above the follicular phase baseline on the day of the LH peak.

Mid-cycle peaks of FSH and LH were not always synchronous and often differed by one day. Transient rises in oestradiol were not usually followed by LH release, even though these

transient increases were sometimes large (Subject KB, Fig. 4. VII). Only when a sustained rise in oestradiol occurred was LH released. The mid-cycle rise in FSH did not begin until the oestradiol rise was established.

Levels of each hormone varied greatly from cycle to cycle and even in the same individual in successive cycles. The normal range for each hormone was therefore large. Patterns of the three steroid hormones were often similar in the luteal phase, suggesting that they were all produced by the corpus luteum but the relative levels of the three hormones were often very different. For example, in subject KB (Fig. 4. VII) and subject KK (Fig. 4. IX), all three hormones were high in the luteal phase; in subject EP (Fig. 4. X) oestradiol was high but 17α-hydroxyprogesterone and progesterone were low; in subject MMc (Fig. 4. XI) and subject PH, cycle 3 (Fig. 4. XIV), progesterone was high whilst 17α-hydroxyprogesterone and oestradiol were low.

The oestradiol rise in the luteal phase was usually about 50% of the mid-cycle rise. The BBT curve was always bi-phasic, the temperature increase becoming apparent with increasing production of progesterone.

The areas under the luteal phase curves for progesterone, 17α-hydroxyprogesterone and oestradiol are compared to their

peak heights in Table 4.2. For each hormone there was good correlation between peak height and area; in general the greater the peak heights the greater the area under the curves. However, both these factors appeared to be independent of the length of the luteal phase.

The results of hormone assays in the normal cycle thus showed great variability both in pattern and level. The results for cycles which were not considered 'normal' in terms of this study can now be examined in the light of this broad outline of 'normal' characteristics.

PART II

HORMONE PATTERNS IN 'ABNORMAL' MENSTRUAL CYCLES

In this section, hormone patterns are studied in cycles which were not considered to be entirely normal and were therfore not included in the normal series. The cycles presented are divided into two groups :-

1. Cycles which show certain abnormalities, although obtained from apparently normal subjects.

2. Cycles from subjects who have some special condition or circumstances.

TABLE 4. 2. COMPARISON OF PEAK LEVELS OF PROGESTERONE, 17α-HYDROXYPROGESTERONE, AND OESTRADIOL WITH THE AREAS UNDER THEIR CURVES IN THE LUTEAL PHASE OF NORMAL MENSTRUAL CYCLES

SUBJECT	LUTEAL PHASE (DAYS)	P		17 OHP		E_2	
		AREA (ng)	PEAK HEIGHT (ng)	AREA (ng)	PEAK HEIGHT (ng)	AREA (pg)	PEAK HEIGHT (pg)
EP	13	39.3	6.3	11.1	1.3	3000	390
KB (Cycle 1	13	70.2	11.4	34.6	4.6	3150	325
MF	14	68.2	8.0	24.7	2.7	1865	300
PH (Cycle 1)	14	56.6	7.2	33.1	3.6	2830	305
PH (Cycle 2)	14	69.6	8.3	19.5	2.7	1220	145
PH (Cycle 3)	14	103.1	13.4	12.7	1.6	1200	125
MMc	15	68.4	10.3	8.9	1.1	1565	170
KK	17	102.8	11.5	48.5	4.9	3270	340

Group 1 consists of :-

a) Cycles from subjects MA, BW (2 cycles, although not consecutive) and CW, all having luteal phases of less than 13 days.

b) 2 cycles from subject JG (not consecutive) and one from subject SH, which have short luteal phases and/or certain other abnormalities.

Group 2 consists of :-

a) A cycle from subject SR who had an intra-uterine device inserted immediately prior to this cycle.

b) A cycle from subject KB, after termination of pregnancy.

c) A cycle from subject HG, who became ill during the cycle and took regular doses of an aspirin containing compound for several days during the luteal phase.

d) A cycle from subject DB. This cycle was considered abnormal because of the magnitude of the LH peak, which was almost twice the value of those found in the 'normal' cycles.

e) Samples from subject PH during a period of amenorrhoea presumably due to anorexia nervosa.

GROUP 1 (a)

Subject MA - The results are shown in Fig. 4. XV. This cycle had an 11 day luteal phase. Oestradiol levels were low in the early follicular phase and they rose consistently from day -5, reaching a plateau on day -1 and 0. Levels dropped on day +1 and a luteal rise began on day +2, culminating in a peak on day +6. A discrete mid-cycle 17α-hydroxyprogesterone peak occurred on day 0. An early peak also occurred in the follicular phase on day -11 but this was not associated with a rise in oestradiol or LH. The BBT curve was bi-phasic. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase were 65.4 ng, 30.1 ng and 1730 pg respectively. FSH results were not available for this cycle.

Subject BW (Cycle 1) - The results are shown in Fig. 4. XVI. This cycle had a 12 day luteal phase. FSH levels began low and rose to a peak on days -14 and -13. Peaks of oestradiol and 17α-hydroxyprogesterone also occurred on day -14. FSH levels dropped on day -10 and reached peak values again on day -8. A rise in progesterone also occurred on day -8. Levels of FSH

FIG. 4. XV STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A 'SHORT' LUTEAL PHASE CYCLE.
SUBJECT M.A.

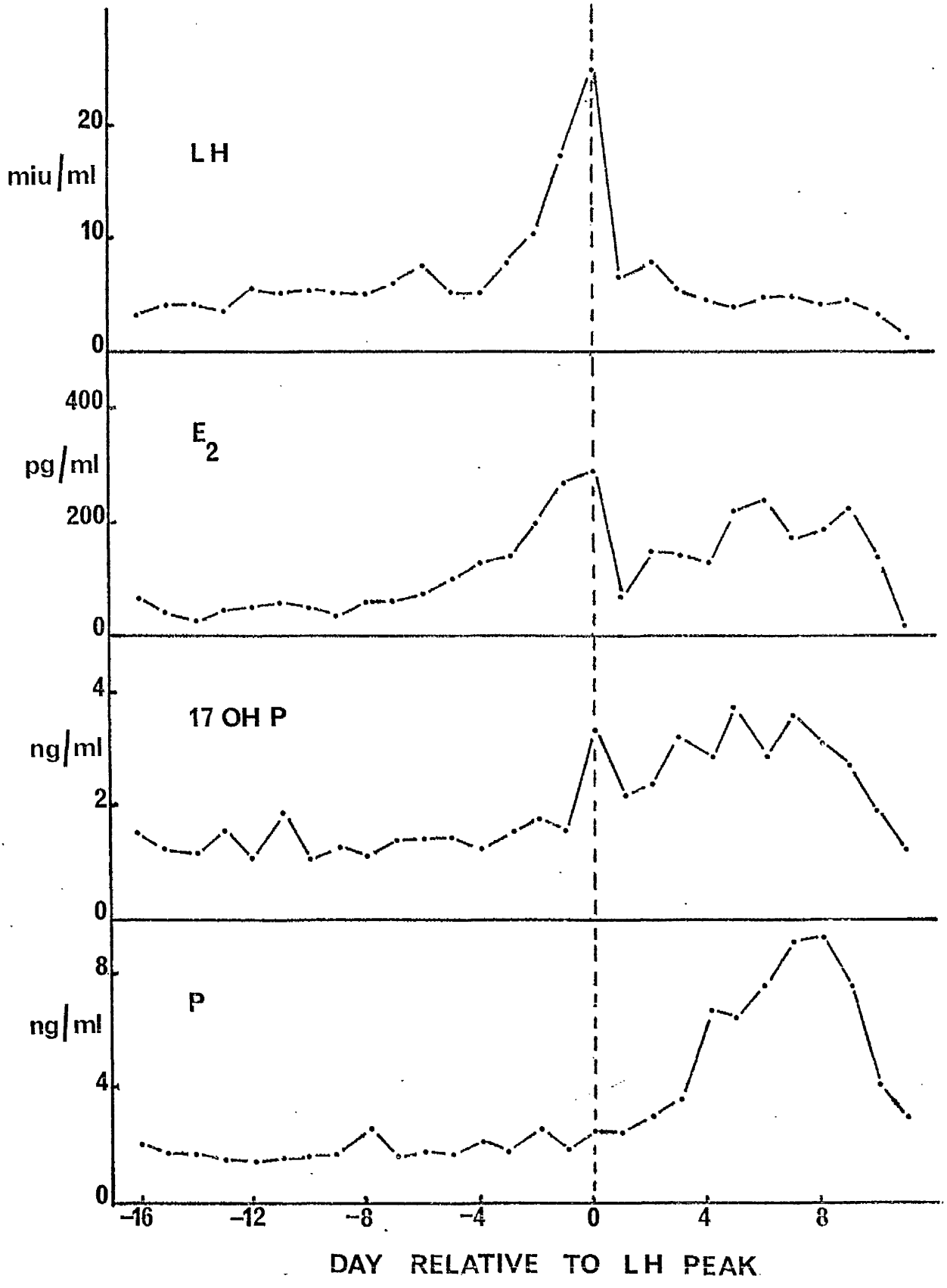
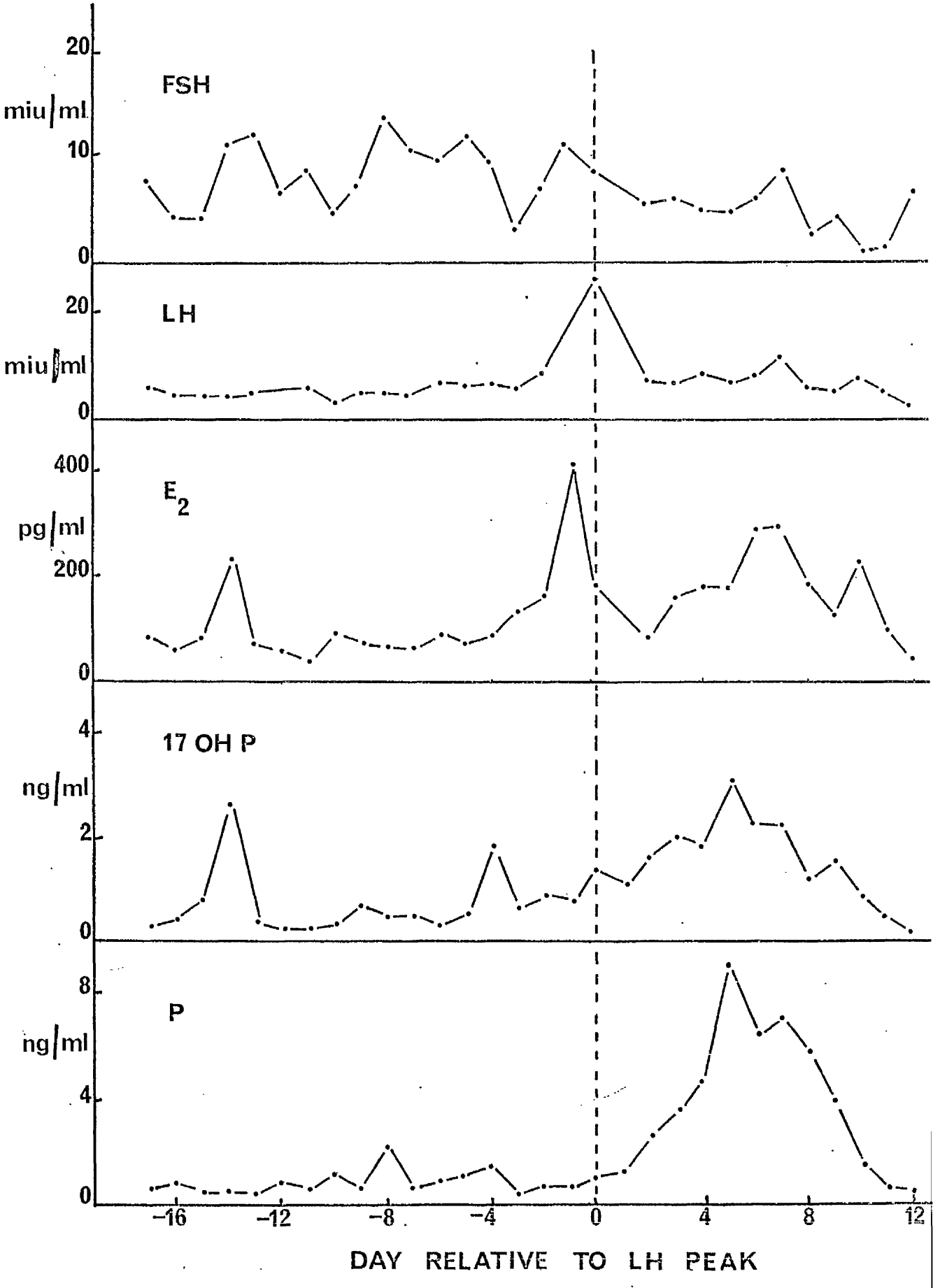


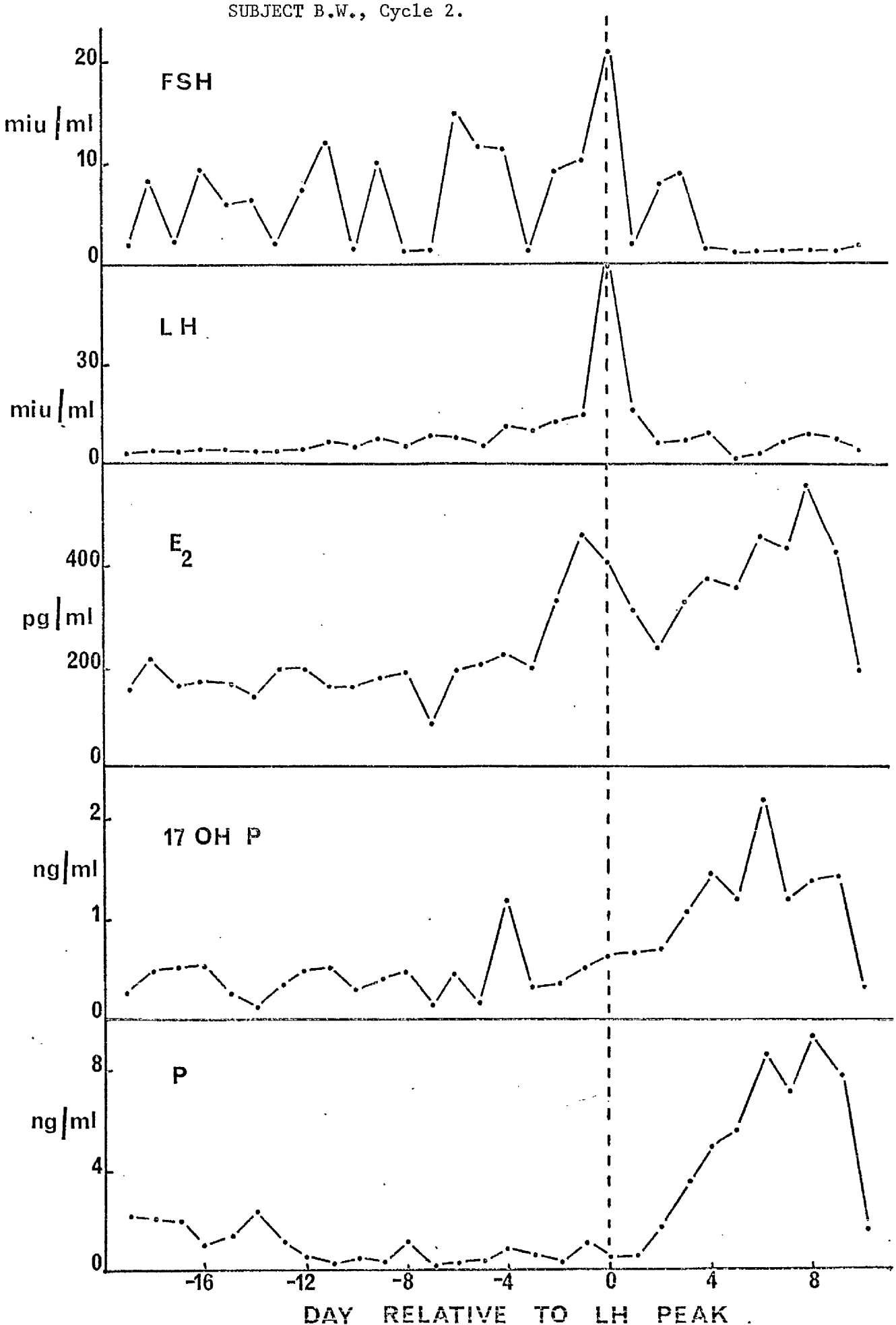
FIG. 4. XVI STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A 'SHORT' LUTEAL PHASE CYCLE.
SUBJECT B.W., Cycle 1.



were still high on day -5 and a peak of 17α-hydroxy-progesterone occurred on day -4. A rise in progesterone occurred between days -7 and -4. The mid-cycle FSH and oestradiol peaks occurred on day -1. A rise in 17α-hydroxyprogesterone began on day 0 and continued into the luteal phase. The BBT curve was biphasic. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase were 46.5 ng 18.5 ng and 1855 pg respectively.

(Cycle 2) - The results are shown in Fig. 4. XVII. This cycle had a 10 day luteal phase. FSH levels fluctuated in the follicular phase, but these changes were not associated with fluctuations in oestradiol levels. Progesterone levels began relatively high in the early follicular phase but dropped on day -11 to the normal range. The mid-cycle oestradiol peak occurred on day -1 and the mid-cycle FSH peak on day 0. Oestradiol levels reached a peak value before the sharp rise in LH occurred. Both oestradiol and LH were high in relation to the 'normal range' at mid-cycle. Unlike the 'normal' cycle oestradiol levels were higher

FIG. 4. XVII STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A 'SHORT' LUTEAL PHASE CYCLE.
SUBJECT B.W., Cycle 2.

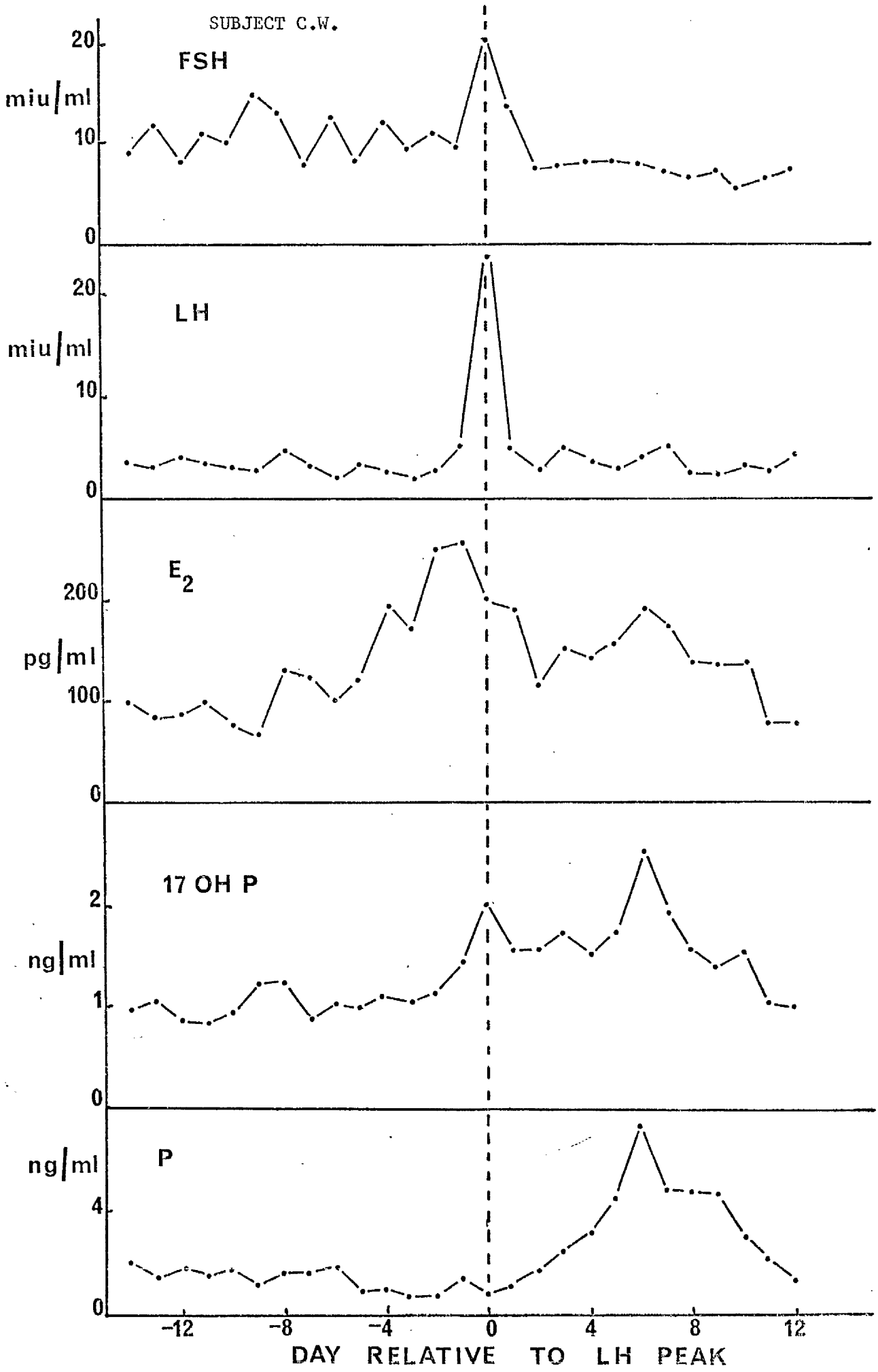


in the luteal phase than at mid-cycle. A peak of 17α-hydroxyprogesterone occurred on day -4 but this was not associated with an increase in oestradiol or progesterone. There was no discrete mid-cycle peak of 17α-hydroxyprogesterone. Patterns of all three steroid hormones were similar in the luteal phase, especially oestradiol and progesterone, which both dropped from their peak luteal values to baseline in two days. The areas under the progesterone, 17α-hydroxyprogesterone, and oestradiol curves in the luteal phase of the cycle were 51.4 ng, 11.3 ng and 3310 pg respectively.

Subject CW - The results are shown in Fig. 4. XVIII. This cycle had a 12 day luteal phase. Progesterone levels were just above the normal range in the early follicular phase, but fell to within the normal range on day -5. Oestradiol reached a mid-cycle plateau on days -2 and -1. The LH and FSH mid-cycle peaks were synchronous. A discrete mid-cycle peak of 17α-hydroxyprogesterone occurred on day 0. The luteal phase patterns of all three steroid hormones were similar, each reaching a peak on day +6. The areas under the

FIG. 4. XVIII STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A 'SHORT' LUTEAL PHASE CYCLE.

SUBJECT C.W.

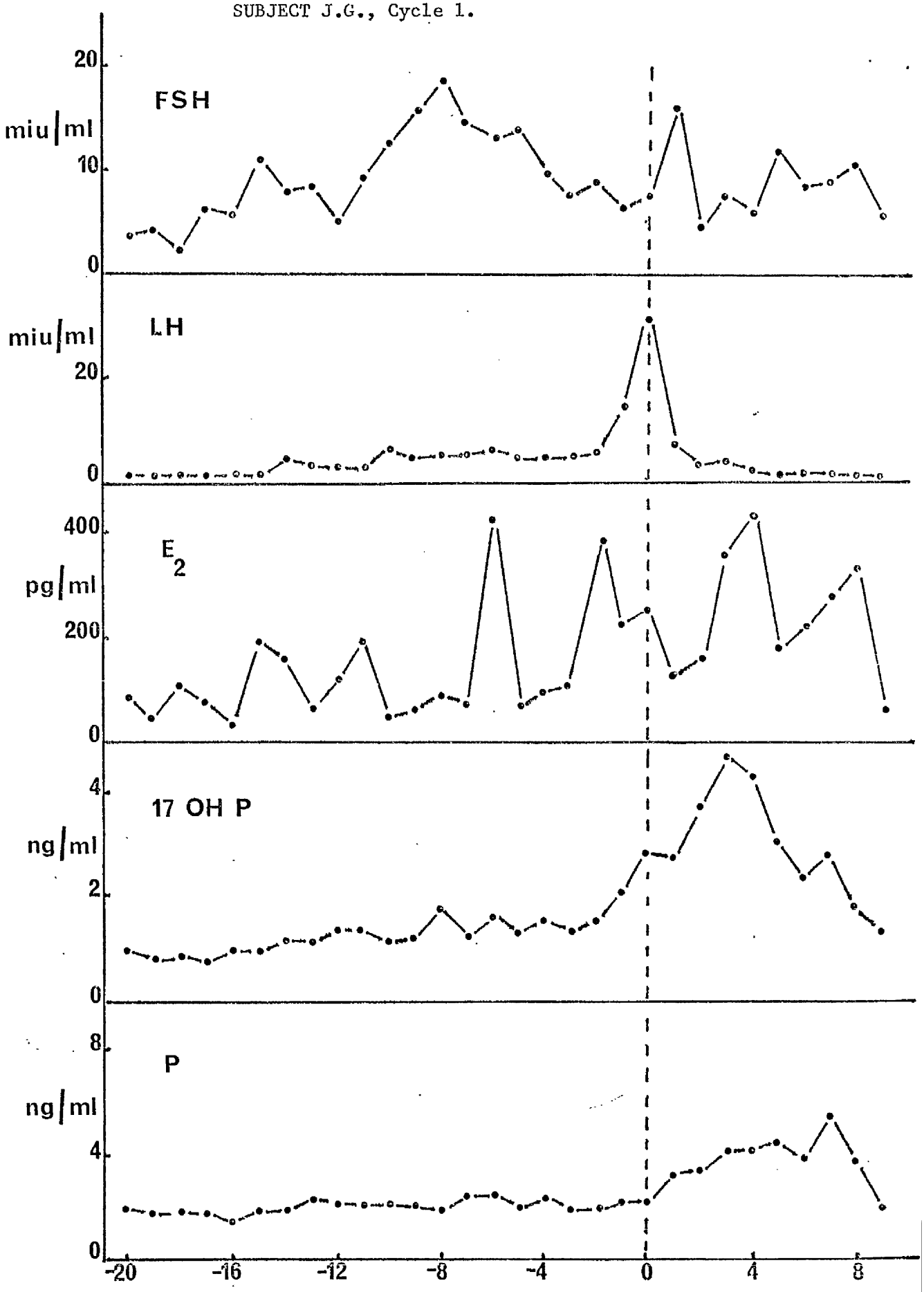


progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase of the cycle were 42.6 ng, 21.4 ng and 1705 pg respectively.

GROUP 1 (b)

Subject JG (Cycle 1) - The results are shown in Fig. 4. XIX. This cycle had a 9 day luteal phase and had several interesting features. Oestradiol and FSH levels fluctuated markedly throughout the cycle. A peak of FSH on day -15 was accompanied by an oestradiol peak, and a peak of FSH on day -8 was followed by a sharp rise in oestradiol on day -6. This oestradiol peak was not followed by a rise in LH. However, a further sharp rise in oestradiol to a peak of equal magnitude (day -2) was followed by a rise in LH. This rise in LH was in fact not apparent until oestradiol levels were dropping. A sustained high level of oestradiol did not, in this cycle, precede LH release. The mid-cycle FSH peak fell on day +1 and was of equal magnitude to the follicular phase peak on day -8. 17α-hydroxy-progesterone rose by day 0 and the rise was sustained into the luteal phase. Peak oestradiol levels in the luteal phase were higher in relation to the mid-cycle

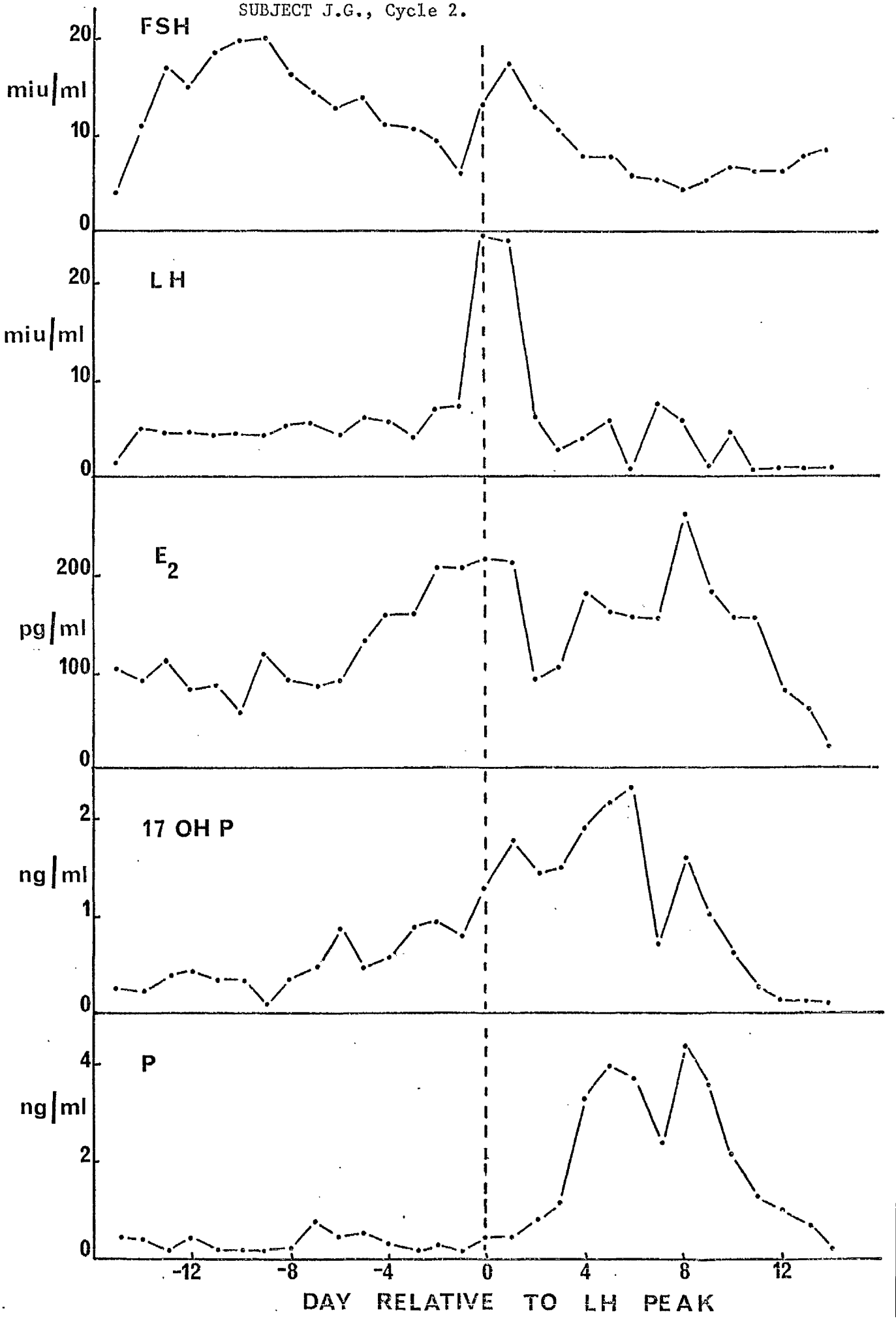
FIG. XIX STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A 'SHORT' LUTEAL PHASE CYCLE.
SUBJECT J.G., Cycle 1.



peak than in the normal cycle. A sharp drop occurred on day +5 followed by a second luteal peak on day +8. A similar drop occurred in 17α-hydroxyprogesterone levels. Progesterone levels were above the normal range throughout the follicular phase of the cycle and fell just within the lower limits of the range in the luteal phase. Levels of oestradiol and 17α-hydroxy-progesterone on the other hand were high in the luteal phase. The BBT curve showed a bi-phasic pattern although the temperature increase occurred on day -6 and was not associated with a rise in progesterone levels. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase of the cycle were 37.3 ng, 27.2 ng and 2150 pg respectively.

(Cycle 2) - The results are shown in Fig. 4. XX. This cycle had a normal length luteal phase (14 days) but showed certain other abnormalities when compared to the normal cycle. Oestradiol levels rose consistently from day -6 and reached a mid-cycle plateau from day -2 to day +1. An FSH mid-cycle peak, of equal magnitude to follicular phase levels, occurred on day +1. 17α-

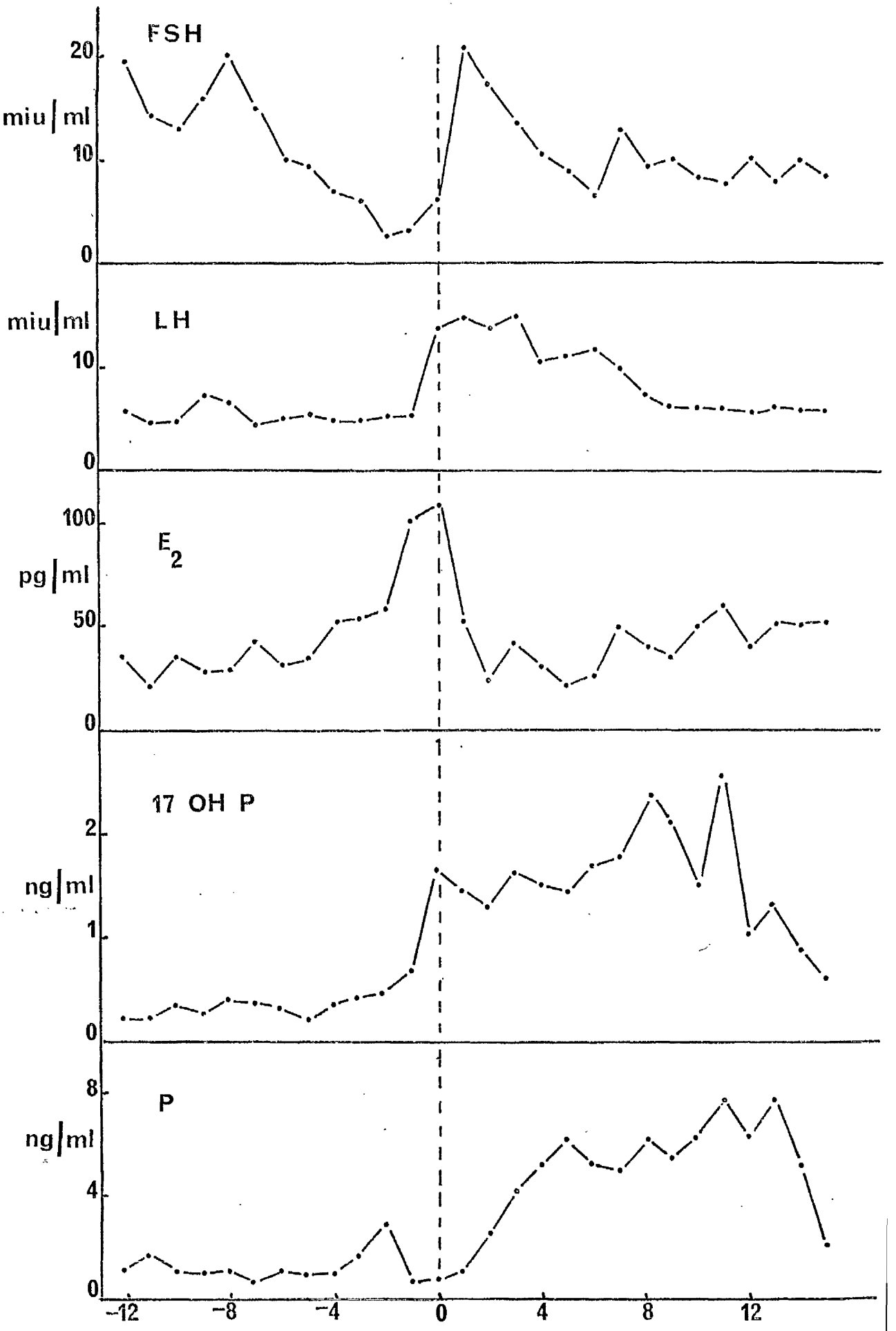
FIG. 4. XX STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A CYCLE SHOWING CERTAIN ABNORMALITIES.
SUBJECT J.G., Cycle 2.



hydroxyprogesterone levels rose by day +1 and the rise continued into the luteal phase. Peak LH levels were sustained for two days. Progesterone levels were below the normal range in both the follicular phase and the luteal phase. Oestradiol levels were higher in the luteal phase, in relation to mid-cycle levels than in the normal cycle. A sharp drop occurred in both progesterone and 17α-hydroxyprogesterone levels on day + 7. The areas under the progesterone, 17α-hydroxy-progesterone and oestradiol curves in the luteal phase of the cycle were 29.1 ng, 15.1 ng and 1790 pg respectively.

Subject SH - The results are shown in Fig. 4. XXI. This cycle was considered to be abnormal because the mid-cycle LH 'peak' extended over 4 days. It was therefore difficult to calculate the length of the luteal phase. The day of the first LH elevation was designated as day 0. FSH levels were high in the follicular phase and dropped by day -2, whence oestradiol levels rose to a mid-cycle plateau on days -1 and 0. The mid-cycle FSH peak occurred on day +1. A significant rise in 17α-hydroxyprogesterone occurred on day 0 and was continued

FIG. 4. XXI STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A CYCLE WITH AN ABNORMAL LH MID-CYCLE 'PEAK'.
SUBJECT S.H.

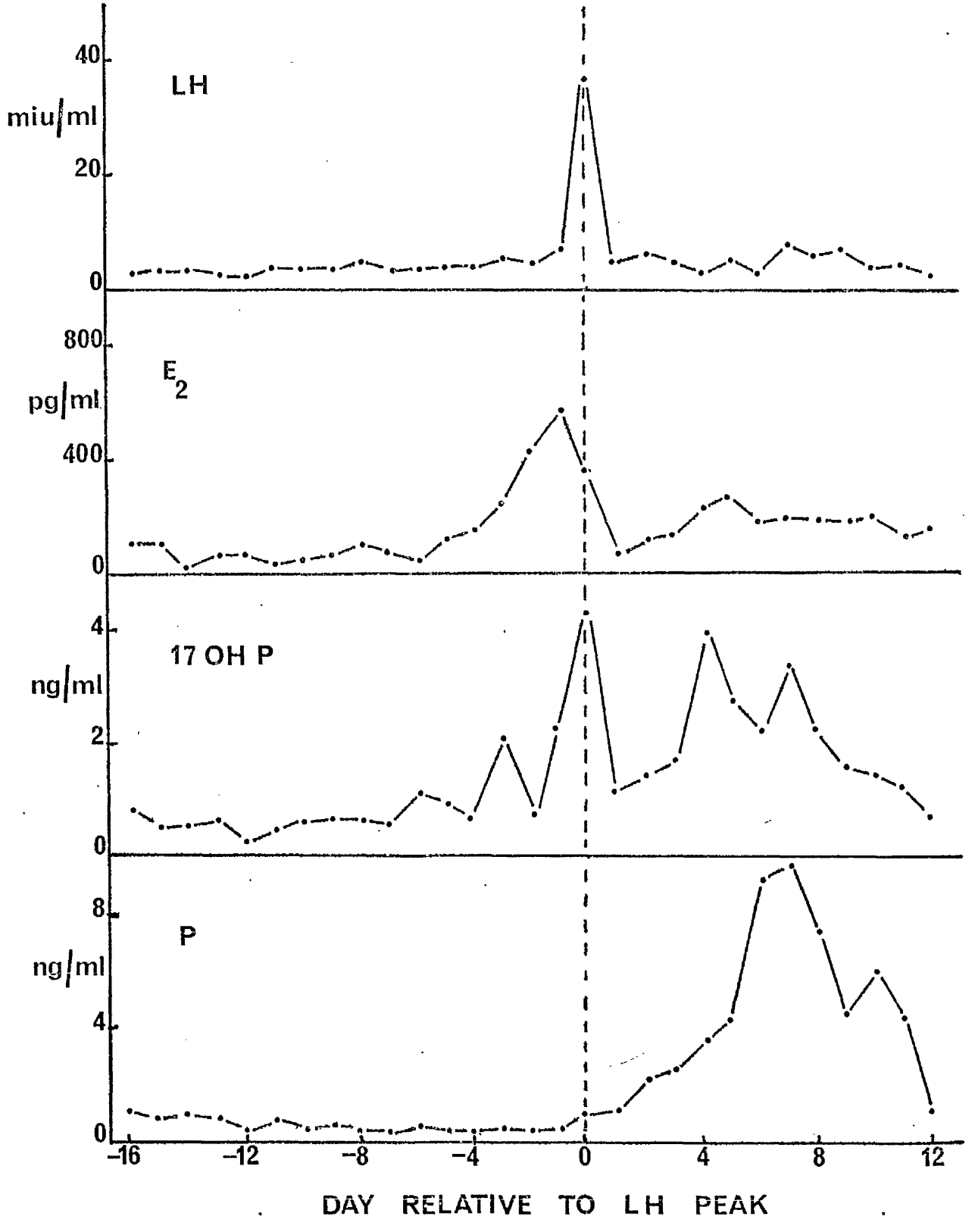


into the luteal phase. A rise in progesterone levels occurred on day -2, before the first rise in LH became apparent. The progesterone levels dropped to baseline before the luteal phase rise began. The LH levels at mid-cycle were below the normal range, as were oestradiol levels at mid-cycle and in the luteal phase. Progesterone and 17α-hydroxyprogesterone levels fell within the normal range throughout the cycle. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase were 77.3 ng, 22.2 ng and 590 pg respectively.

GROUP 2

a) Subject SR - This subject had an intra-uterine contraceptive device inserted immediately prior to the cycle in which samples were collected. The results are shown in Fig. XXII. The cycle had a 12 day luteal phase. The mid-cycle oestradiol peak occurred on day -1. Peak levels of both LH and oestradiol were above the normal range. A peak of 17α-hydroxyprogesterone occurred on day -3, and a mid-cycle peak, above the normal range, occurred on day 0. Progesterone levels fell within the normal range throughout the cycle. The BBT curve was biphasic, the temperature increase being apparent by day +4. The areas under the

FIG. 4. XXII STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A CYCLE FROM A SUBJECT WITH AN INTRA-UTERINE DEVICE. SUBJECT S.R.



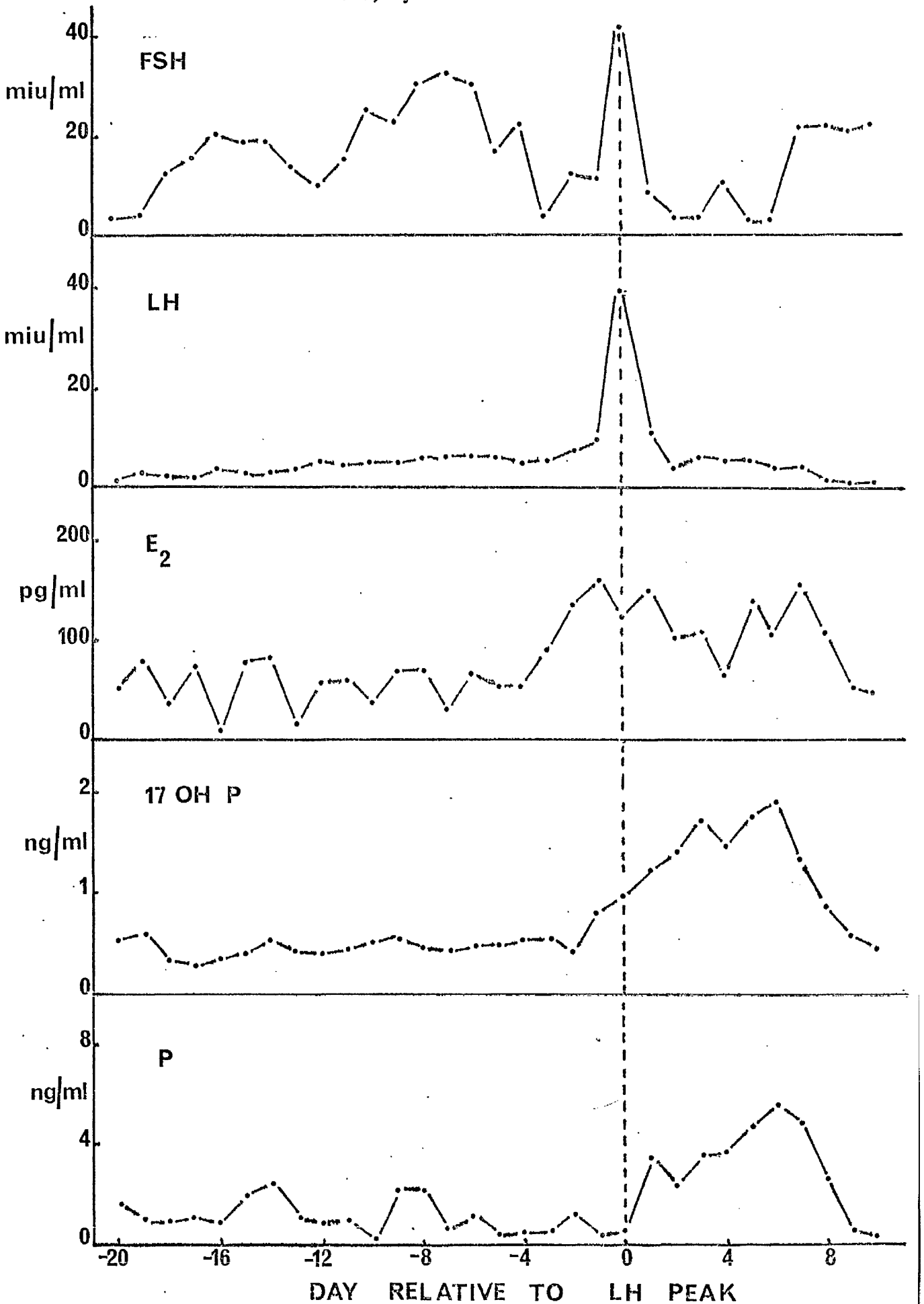
progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase of the cycle were 58.2 ng, 24.3 ng and 2075 pg respectively.

b) Subject KB (Cycle 2) - This subject, who was studied in Part I of this chapter, subsequently became pregnant and was therefore the only one of proven fertility. Pregnancy was terminated at 12 weeks gestation by extra-amniotic infusion of prostaglandin E_2. Five months after the termination, when normal menstruation had been resumed for three months, daily blood samples were collected throughout a cycle.

No systemic study appears to have been undertaken of comparison of plasma hormone patterns throughout cycles from nulliparous and parous women. Information is therefore not available as to whether pregnancy alters hormone production during ensuing cycles either in peak values or in patterns. This cycle was not considered for inclusion in the normal series, but was interesting to examine with regard to the possible effect of pregnancy and prostaglandin E_2 administration on subsequent menstrual cycles.

The results are shown in Fig. 4. XXIII. Although of 32 days duration the cycle had only a 10 day luteal phase.

FIG. 4. XXIII STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A CYCLE AFTER TERMINATION OF PREGNANCY.
SUBJECT K.B., Cycle 2.



Oestradiol levels began to rise, at mid-cycle from day -3 onwards and reached maximum levels on day -1. No drop occurred after this, as in the normal cycle, but elevated levels continued into the luteal phase, until day +4 when levels dropped to 50% of the peak value. A peak of equal magnitude to the mid-cycle peak occurred on day +7. 17α-hydroxyprogesterone levels rose significantly by day 0, the rise continuing into the luteal phase. Two peaks of progesterone occurred in the follicular phase on days -14 and -9. Levels of all steroid hormones were low in the luteal phase, compared to the mean normal cycle. The FSH mid-cycle peak occurred on day 0. FSH and LH levels at mid-cycle were above the normal range, whilst oestradiol levels were below the normal range. In the luteal phase, FSH levels rose on day +7 before progesterone and oestradiol levels fell. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase of the cycle were 32.1 ng, 12.8 ng and 1.015 pg respectively.

c) Subject HG - This subject became ill during the cycle in

which blood was collected. Large doses of an aspirin containing compound were administered from days +2 to +6, inclusive. The results are shown in Fig. 4. XXIV. The baseline levels of the steroid hormones were low in the follicular phase, and FSH levels were also low during this time. A peak of oestradiol occurred on day -13. Mid-cycle levels of oestradiol were low, a peak occurring on day 0. LH and FSH mid-cycle peaks were synchronous and above the normal range. Luteal phase levels of progesterone and 17α-hydroxyprogesterone were below the normal range. No increase in FSH occurred towards the end of the cycle. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase of the cycle were 50.0 ng, 8.0 ng and 990 pg respectively.

d) Subject DB - This cycle is shown in Fig. 4. XXV. Mid-cycle peaks of FSH and LH were synchronous and both were well above the normal range, especially LH which was twice as high as peaks found in normal cycles. The mid-cycle peak of oestradiol however was below the normal range, and occurred on day -1. FSH levels were within the normal range in the follicular phase, but were accompanied by low levels of oestradiol. A rise of 17α-hydroxyprogesterone

FIG. 4. XXIV STEROID AND GONADOTROPHIN HORMONE PATTERNS IN A CYCLE DURING WHICH ASPIRIN WAS ADMINISTERED FROM DAY +2 TO +6 INCLUSIVE

SUBJECT H.G.

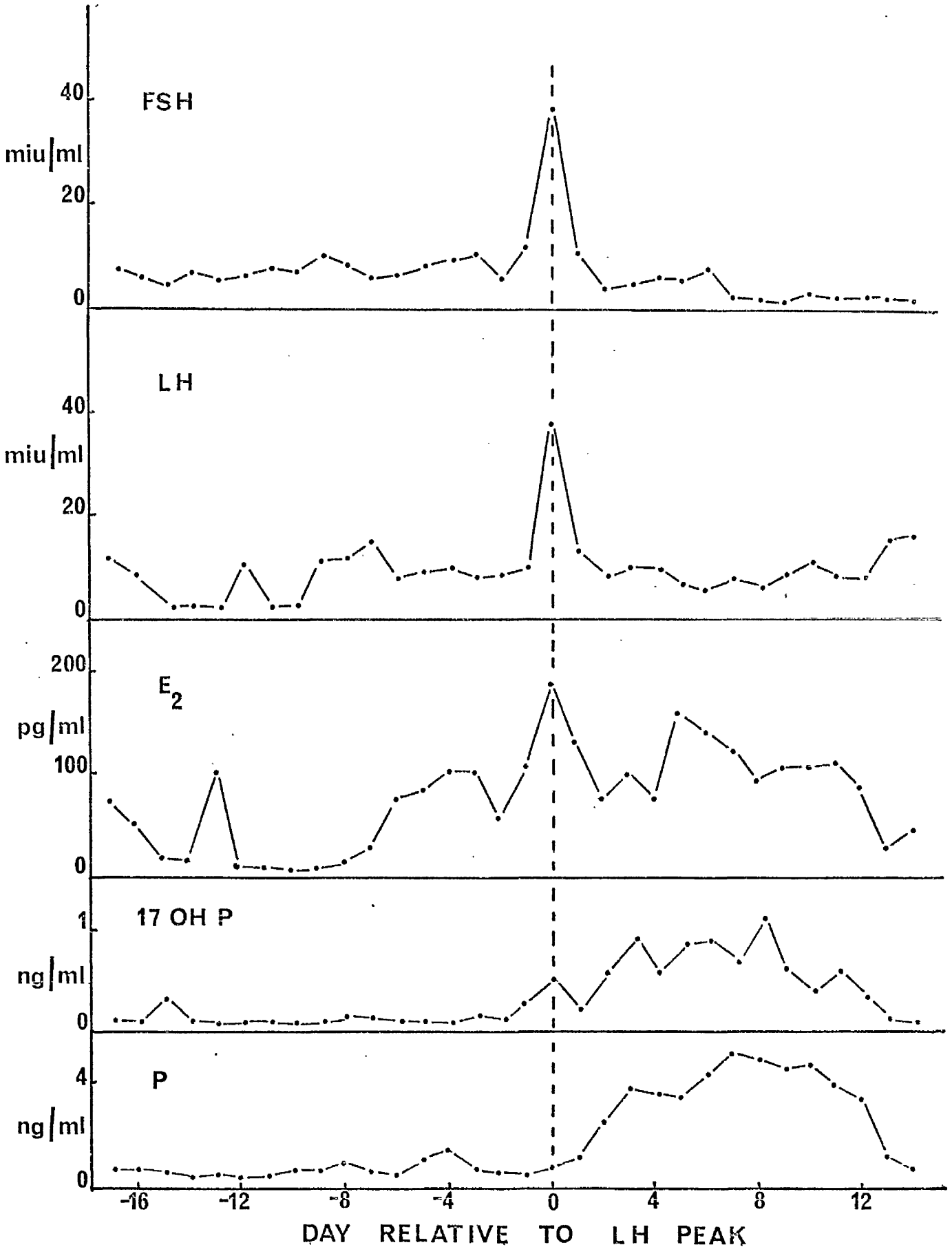
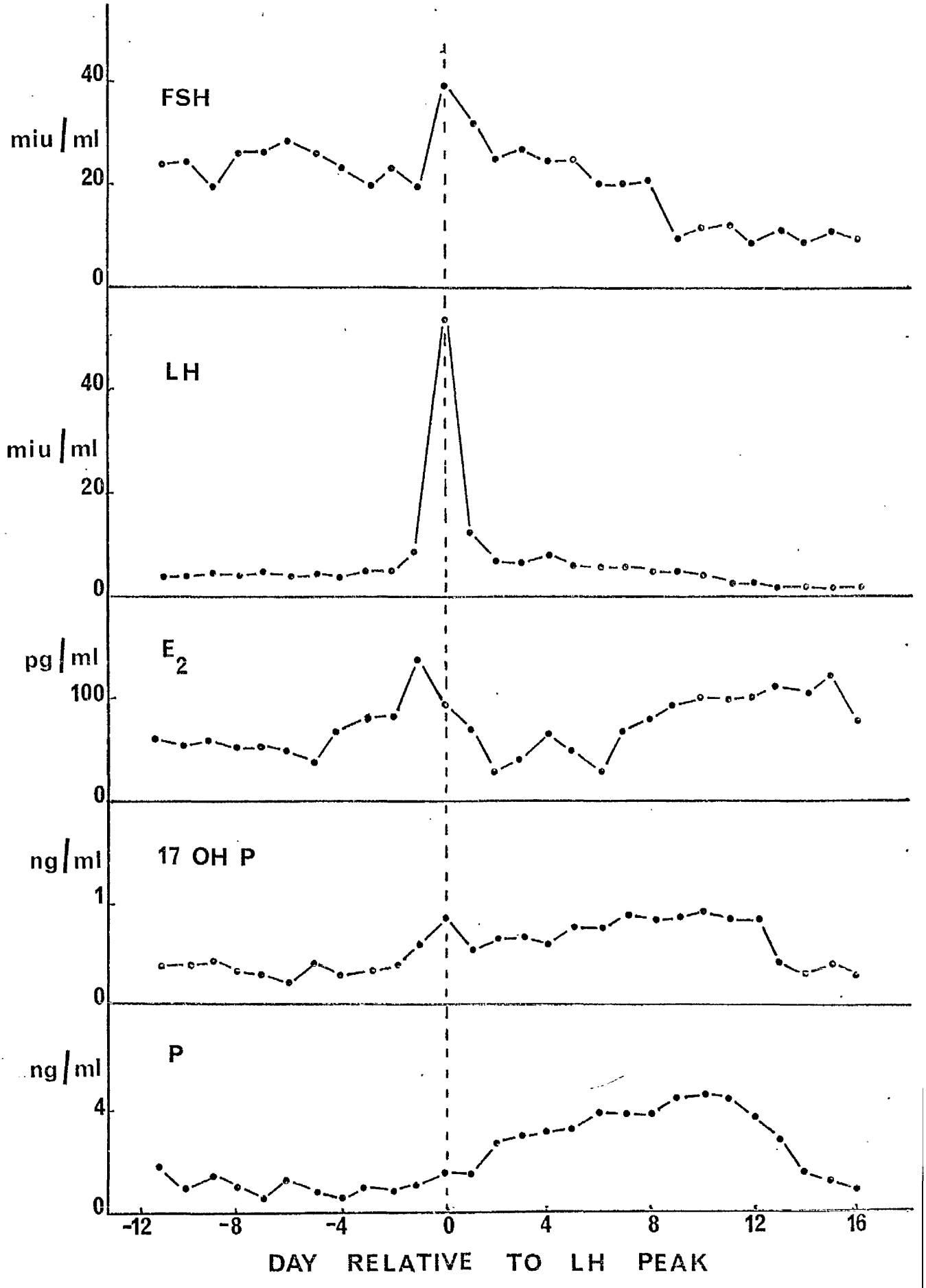


FIG. 4. XXV STEROID AND GONADOTROPHIN HORMONE PATTERNS THROUGHOUT A CYCLE WITH AN ABNORMALLY HIGH LH PEAK SUBJECT D.B.



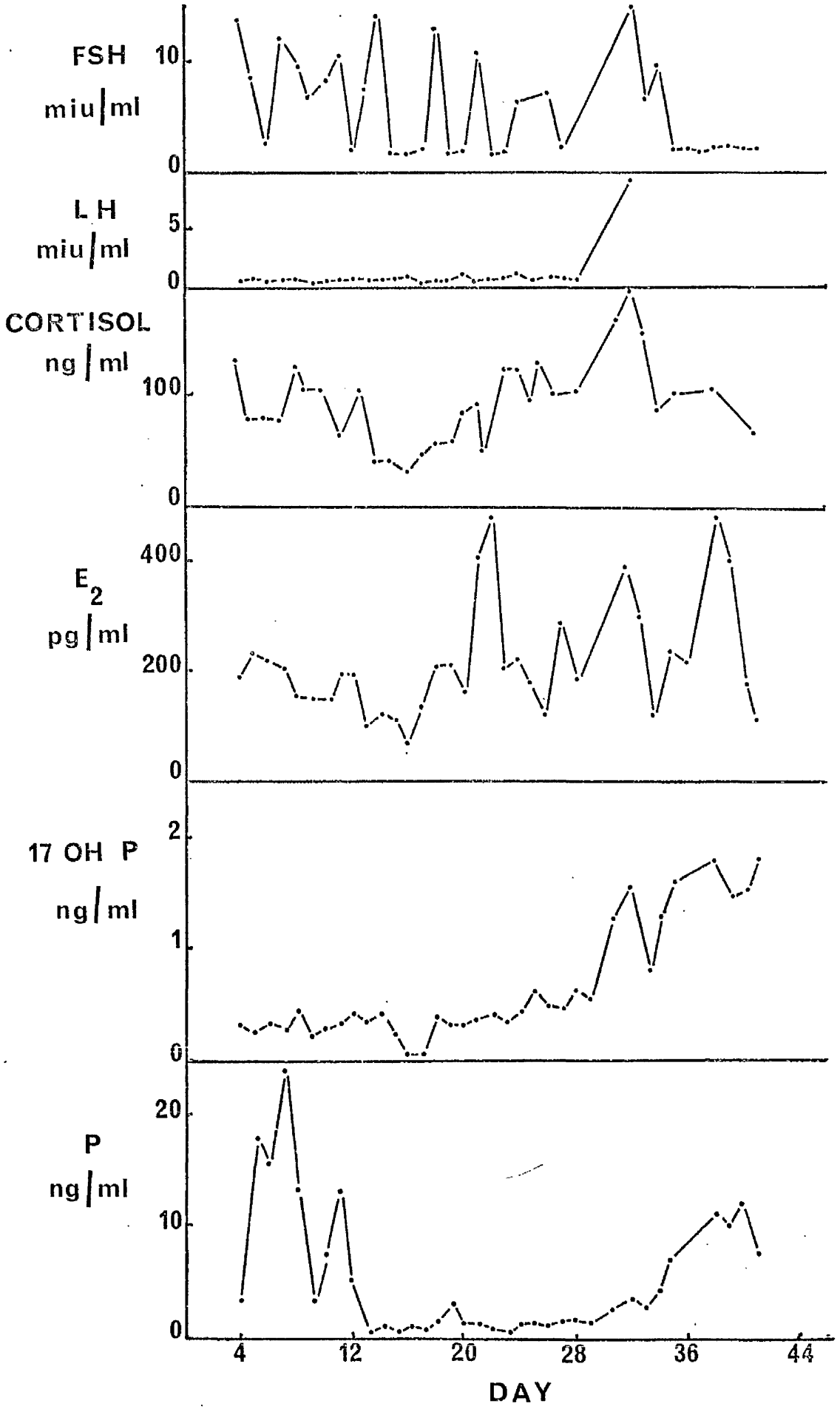
occurred on day 0. Levels of all three steroid hormones were below the normal range in the luteal phase of the cycle. The areas under the progesterone, 17α-hydroxy-progesterone and oestradiol curves in the luteal phase of the cycle were 50.1 ng, 9.7 ng and 1165 pg respectively.

e) Subject PH - Blood samples were collected from this subject during a period of amenorrhoea associated with dieting (see p. 74). Cortisol was measured, as described on p. 28 , in these samples, along with the usual steroid and gonadotrophin hormones. The results are shown in Fig. 4. XXVI. The subject had not menstruated for 12 weeks prior to collection of the first blood sample. Menstruation then occurred six days after collection of the last sample.

Very high levels of progesterone were found at the beginning of the sampling period, but the levels dropped to the follicular phase range by the 13th day. Levels rose again from day 30 to reach levels similar to those found in the luteal phase of a normal cycle by the end of the collection period. 17α-hydroxyprogesterone levels were low and did not fluctuate until day 32 when a peak occurred. A

FIG. 4. XXVI STEROID AND GONADOTROPHIN HORMONE PATTERNS THROUGHOUT A PERIOD OF AMENNORHOEA, PRESUMABLY DUE TO ANOREXIA NERVOSA.

SUBJECT P.H.



drop occurred on day 31 followed by a further rise coincident with the progesterone rise. The levels of 17α-hydroxyprogesterone were similar to those found at mid-cycle and in the luteal phase of a normal cycle. Oestradiol levels were around 200 pg/ml. at the beginning of the collection period and dropped gradually to 60 pg/ml on day 16. A peak occurred (500 pg/ml) on day 22 and further peaks on day 27 (300 pg/ml), 32 (400 pg/ml) and 38 (500 pg/ml). The peak on day 27 coincided with the 17α-hydroxyprogesterone peak and that on day 38 with the progesterone and 17α-hydroxyprogesterone rises.

LH levels were very low until day 29 when they rose to 9 m.i.u./ml on day 31. Unfortunately LH results were not available for the remaining days.

FSH levels were within the normal range throughout the collection period and fluctuated greatly. A peak of similar magnitude to the mid-cycle peak in the normal cycle occurred on day 31, coincident with the LH rise. Levels then dropped and remained steady for the remaining days of the collection period.

Cortisol levels were within the normal range throughout (Fraser, 1973).

TABLE 4. 3. COMPARISON OF 'PEAK' HEIGHTS OF PROGESTERONE, 17α-HYDROXYPROGESTERONE AND OESTRADIOL WITH THE AREAS UNDER THEIR CURVES IN THE LUTEAL PHASE OF 'ABNORMAL' MENSTRUAL CYCLES

SUBJECT	LUTEAL PHASE (DAYS)	P		17 OHP		E_2		COMMENTS
		AREA (ng)	PEAK HEIGHT (ng)	AREA (ng)	PEAK HEIGHT (ng)	AREA (pg)	PEAK HEIGHT (pg)	
MA	11	65.4	9.3	30.1	3.7	1730	240	Short luteal phase
BW 1	12	46.5	9.0	18.5	3.1	1855	290	"
2	10	51.4	9.5	11.3	2.2	3310	550	"
CW	12	42.6	7.5	21.4	2.6	1705	190	"
JG 1	9	37.3	5.7	27.2	4.8	2150	440	"
2	14	29.1	4.3	15.1	2.3	1790	260	Abnormal E_2 pattern
SH	15*	77.3	7.9	22.2	2.7	590	60	Abnormal LH 'peak'
SR	12	58.2	10.3	24.3	4.1	2075	255	I.U.D. present
KB	10	32.1	4.8	12.8	1.9	1015	155	Post-termination
HG	14	50.0	5.2	8.0	1.1	990	130	Aspirin administration
DB	16	50.1	4.7	9.7	0.84	1165	125	High LH peak

* Calculated from the 1st day of LH rise

SUMMARY OF RESULTS IN 'ABNORMAL' MENSTRUAL CYCLES

A large number of cycles from apparently normal subjects had some abnormality when studied closely. In general short luteal phase cycles appeared normal in most other respects. The cycles from subjects having some special abnormality must each be considered separately in order to correlate hormonal patterns with that abnormality. Full discussion of these cycles will be presented in Chapter 7. The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves are related to luteal peak heights for each of the subjects studied in this section in Table 4.3. Again there was reasonably good correlation between peak height and area under the curve; in general, for each hormone, the greater the peak height, the greater the area under the curve. However, there was no correlation between peak height or area for one hormone and that for another hormone in the same cycle.

CHAPTER 5

COMPARISON OF HORMONE PATTERNS IN MENSTRUAL CYCLES OF INFERTILE AND NORMAL WOMEN

COMPARISON OF HORMONE PATTERNS IN MENSTRUAL CYCLES OF INFERTILE AND NORMAL WOMEN

INTRODUCTION

The previous chapter established that a wide variation exists in hormone levels and patterns in the apparently normal menstrual cycle. From these results it was possible to define a broad set of characteristics which were common to all normal cycles, although many minor features were specific for individual cycles. Subtle differences may exist in hormone patterns between the normal cycle and cycles in women who are infertile for no apparent physiological reason. Investigation of such cycles by comparison with the normal cycle may indicate which features are essential for fertility and may also help to characterise this type of infertility.

Infertile women included in this study were defined as women who had :-

a) been attempting to conceive for at least two years.

b) normal menstrual rhythm.

c) been investigated and found to have ovaries of normal

appearance (by laparoscopy), patent fallopian tubes and secretory endometrium in the second half of the cycle.

d) husbands with sperm counts of not less than 15 million per ml.

The nature of this infertility and hence its treatment, poses a major problem. Anovulatory infertility is more easily diagnosed and its successful treatment has frequently been reported (e.g. MacGregor et al., 1968; Adamapoulos et al., 1972). However, no attempt has been made to classify or diagnose infertility in which ovulation apparently occurs. Established treatment is therefore not currently in use nor can be attempted until some understanding of the condition is achieved.

The aims of this investigation were twofold; firstly to investigate the mechanism of regulation of the menstrual cycle by comparison of normal with infertile cycles and secondly to obtain a fuller understanding of the reason for the infertility and to attempt to treat the condition.

Six women who fulfilled the conditions outlined above were included in the study. Daily blood samples were collected at

09.00 hours each morning from an arm vein, whilst the patients were sedentary, throughout a menstrual cycle. Plasma levels of progesterone, 17α-hydroxyprogesterone, oestradiol, LH and in some cases FSH were determined as previously described. The hormone levels throughout the cycles of these women were compared individually to the normal ranges.

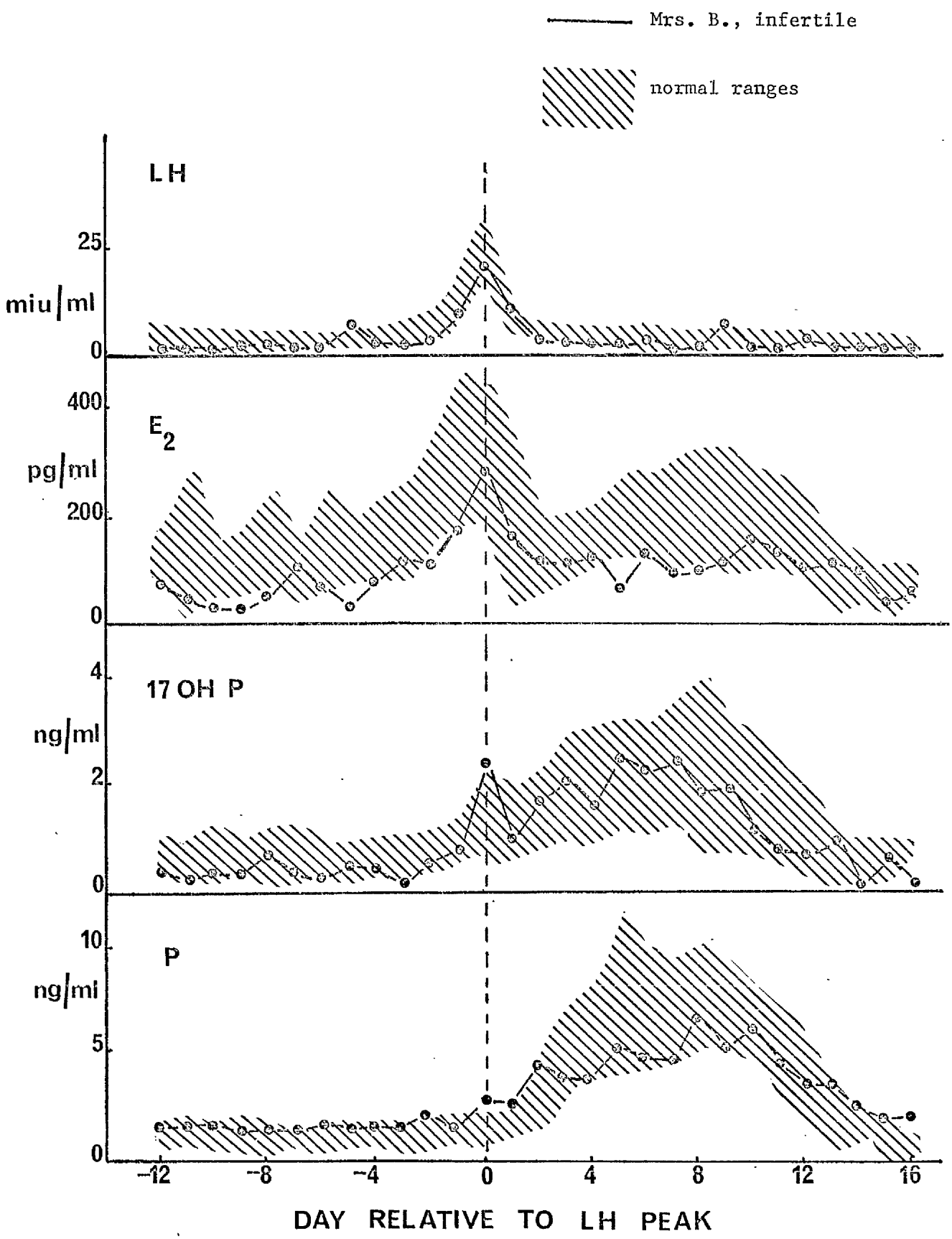
RESULTS

INDIVIDUAL CYCLES

Mrs. B. - This patient was 24 years old, nulliparous and complaining of infertility. She had patent tubes, secretory endometrium and a history of regular menstrual cycles of between 32 and 35 days. Ovarian biopsy on day 19 of her cycle revealed that the capsule of the ovary was slightly thickened, that numerous primordial follicles were present, that two atretic Graafian follicles were present but that there was no evidence of a corpus luteum or corpus albicans to indicate a recent ovulation.

The hormone levels throughout a menstrual cycle were compared to the normal range and are shown in Fig. 5. I. The cycle was of 28 days duration with a 16 day luteal phase.

FIG. 5. I. COMPARISON OF HORMONE PATTERNS IN INFERTILE WOMEN WITH NORMAL MENSTRUAL CYCLE RANGES.



The LH patterns appeared normal in all respects. Levels were within the normal range throughout, although towards the lower limits of this range in the follicular phase and in the late luteal phase. Oestradiol levels, on the other hand, did not show such a normal pattern. Levels were very low in the follicular phase, falling at some points below the normal range. A mid-cycle peak occurred on day 0, the peak value being normal. The LH and oestradiol rises occurred simultaneously. In the luteal phase, oestradiol levels were again low and took longer to reach peak values (day +10) than in the normal cycle. The 17α-hydroxyprogesterone pattern appeared normal; a mid-cycle peak occurred on day 0 of magnitude just within the normal range whilst the luteal rise was similar to the normal range in pattern and levels. Progesterone levels were at the high end of the normal range in the follicular phase. Levels in the luteal phase were at the low end of the normal range, and similarly to oestradiol, took longer to reach peak values (day +8) than in the normal cycle.

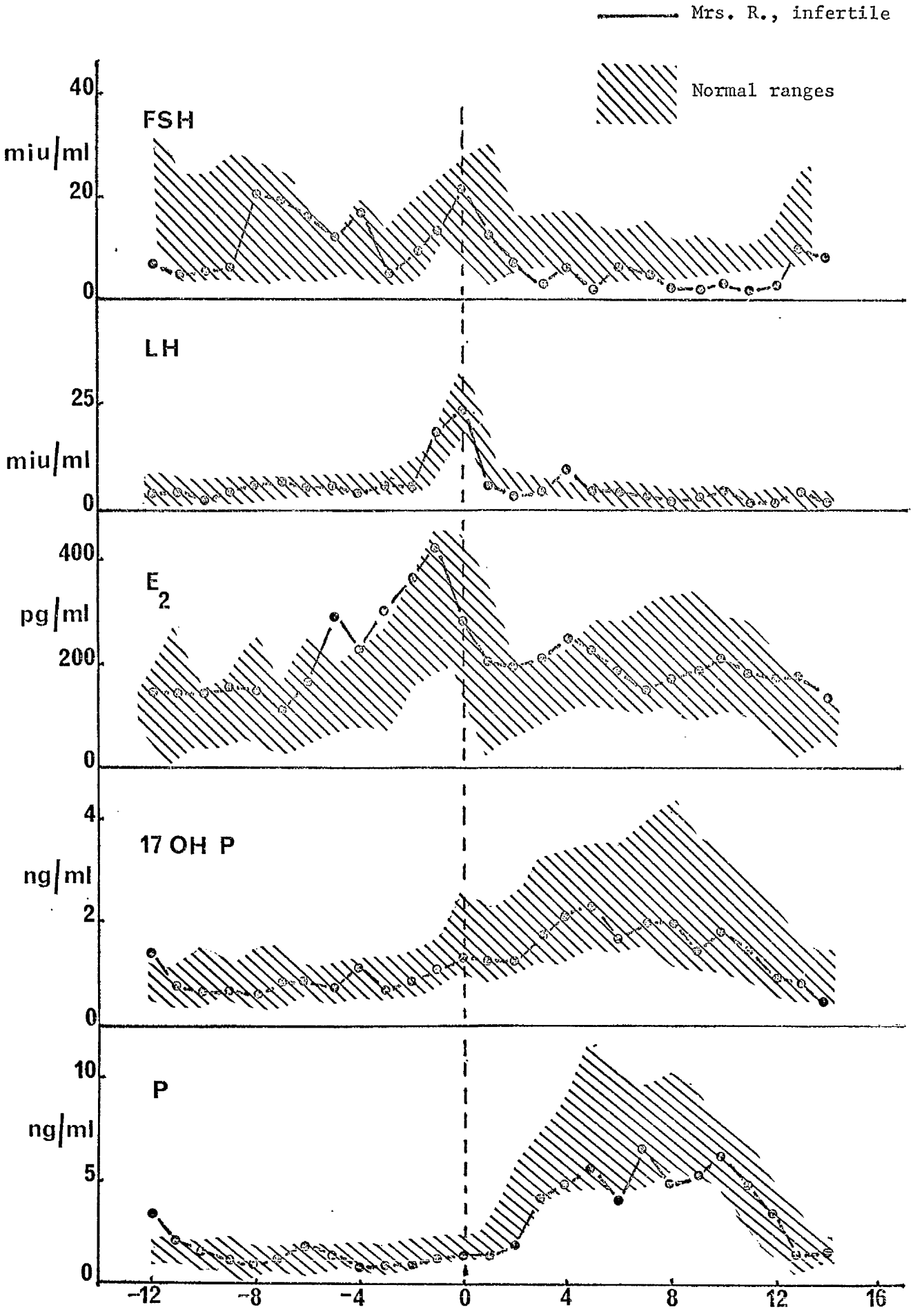
The cycle was apparently ovulatory, on the basis of the LH mid-cycle peak (Abraham et al., 1972) and the luteal phase progesterone rise. However, this cycle was shorter

than the patient's previous history records and on the basis of biopsy reports ovulation had not occurred immediately prior to this cycle.

Mrs. R.- This patient was 25 years old, nulliparous and complaining of infertility of 3 years duration. She had patent tubes, a secretory endometrium and a history of regular menstrual cycles of between 26 and 28 days. The hormone levels throughout a menstrual cycle were compared to the normal ranges and are shown in Fig. 5. II. The cycle was 27 days long with a 14 day luteal phase.

LH levels appeared normal throughout the cycle. FSH levels were low in the early follicular phase (days -12 to -9). The levels and patterns of FSH appeared normal throughout the rest of the cycle, though a steep rise in the late luteal phase did not occur. Oestradiol levels were at the high end of the normal range in the follicular phase and in the pre-ovulatory peak which occurred on day -1. The luteal phase levels appeared normal. 17α-hydroxyprogesterone levels were normal in the follicular phase, but there was no discrete mid-cycle peak or significant rise at mid-cycle. Luteal phase levels were in the normal range. Progesterone levels were above the normal range

FIG. 5. II COMPARISON OF HORMONE PATTERNS IN INFERTILE WOMEN WITH THE NORMAL MENSTRUAL CYCLE RANGES.



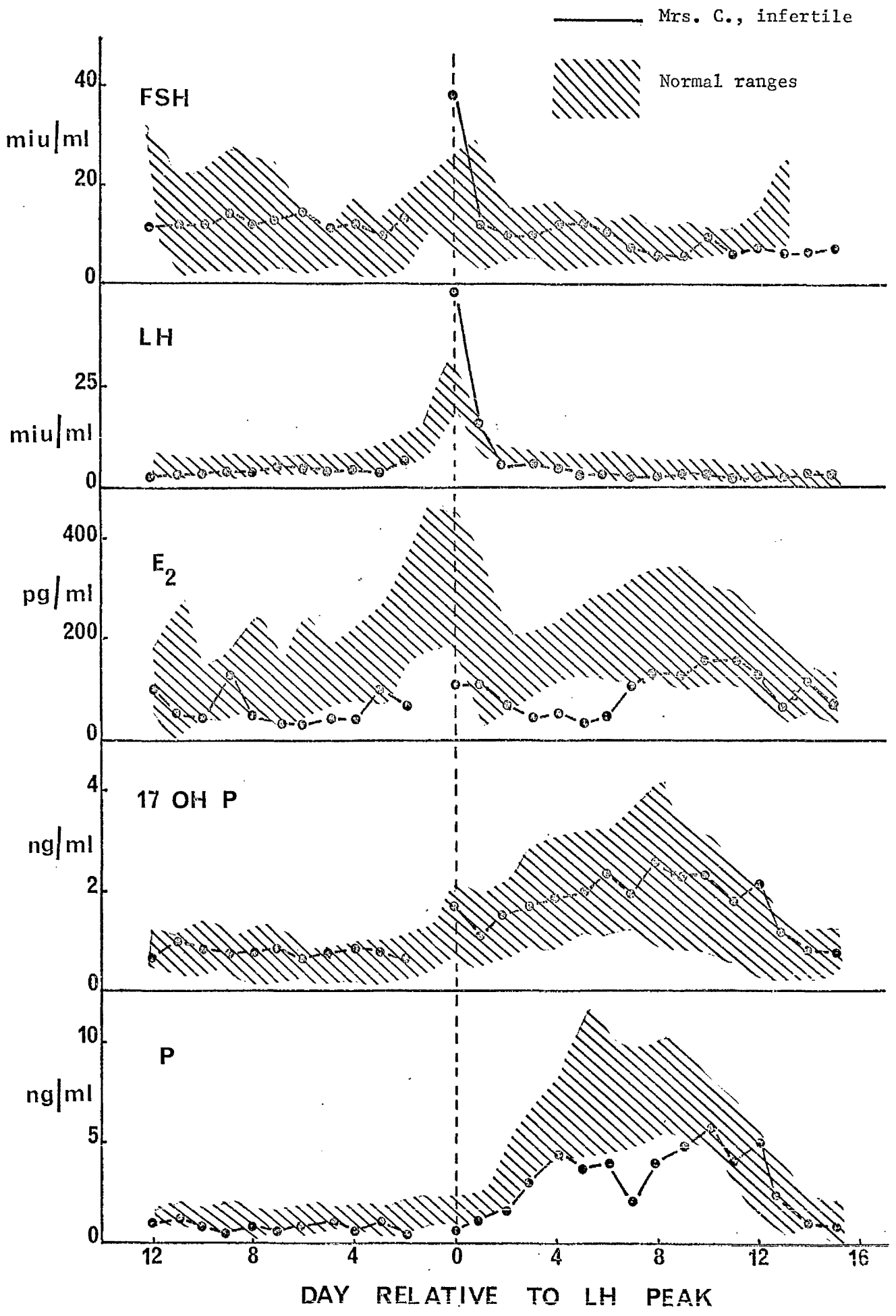
on the first day of the cycle but fell to within the normal range on day -11. Levels were steady until the luteal rise, where levels fell just within the lower limits of the normal range except of days +6 and +8 when they dropped below normal levels. Progesterone levels took longer to reach peak values than in the normal luteal phase.

Ovulation appeared to occur normally in this cycle as judged by the mid-cycle LH surge and luteal progesterone rise.

Mrs. C. - This patient was 32 years old. She was nulliparous and complaining of infertility of six years duration. She had patent tubes, a secretory endometrium (on day 19) and a history of a regular cycle of 28 days. Ovarian biopsy (day 19) revealed several Graafian follicles undergoing atresia and some primordial follicles in the right ovary. A degenerating corpus luteum was present on the left ovary. The hormone patterns throughout a menstrual cycle were compared with the normal range and are shown in Fig. 5. III. The cycle was of 28 days duration with a 15 day luteal phase.

LH levels were normal in the follicular phase but the mid-cycle peak was above the normal range, as was the FSH peak.

FIG. 5. III COMPARISON OF HORMONE PATTERNS IN INFERTILE WOMEN WITH THE NORMAL MENSTRUAL CYCLE RANGES.



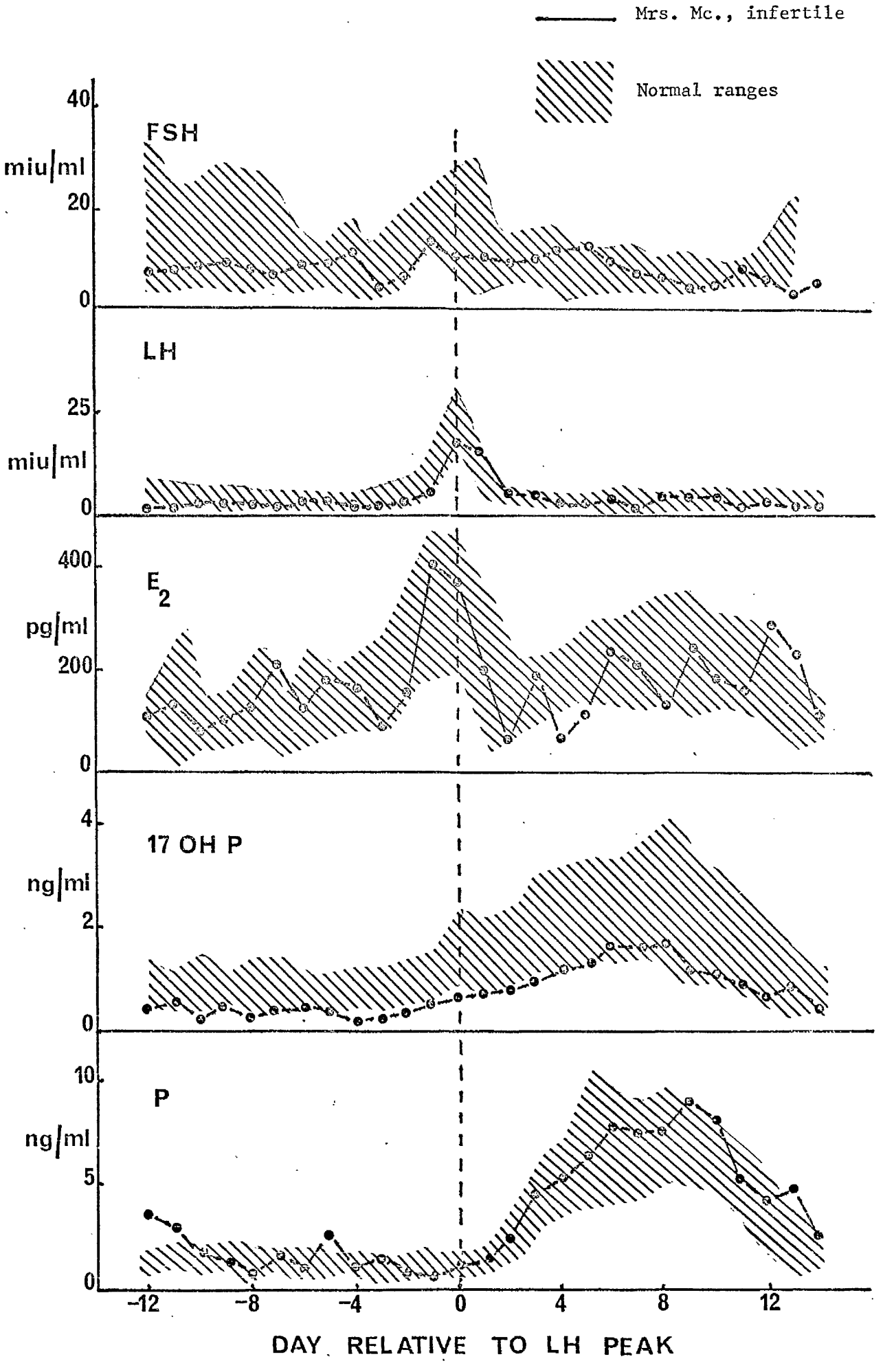
Luteal phase levels of both were normal, though FSH showed no early follicular and late luteal phase elevations. Oestradiol levels were low in the follicular phase and below the normal range from days -8 to -2. The sample on day -1 was not collected, but the oestradiol mid-cycle peak was likely to have occurred on this day. However, levels usually rise at mid-cycle from day -4 onwards and in this case no significant rise had occurred by day -2. Oestradiol levels were well below the normal range in the early luteal phase and reached a late peak (day +10) just within the normal range. 17α-hydroxyprogesterone levels were normal in the follicular phase. A mid-cycle rise was present although the day of the peak could not be identified. Levels fell within the normal range throughout the luteal phase. Progesterone levels were low in the follicular phase but fell just within the normal range on most days. The rate of progesterone increase in the luteal phase was retarded, peak values being reached on day +10. Levels were below the normal range in the early luteal phase. A significant drop occurred on day +7, but levels rose into the normal range in the late luteal phase.

Ovulation appeared to occur in this cycle as judged by the LH mid-cycle surge and the luteal phase progesterone rise.

Mrs. Mc.- This patient was 31 years old and had one child six years previously. She had been attempting to conceive again since that time. Some of her cycles were short (14 days) but the cycle in which she was examined was of 27 days duration. She had patent fallopian tubes and secretory endometrium. Ovarian biopsy on day 14 of her cycle revealed primordial follicles in the ovary but no Graafian follicles. An old corpus albicans was present. The hormone patterns throughout a menstrual cycle were compared to the normal ranges and are shown in Fig. 5. IV. The cycle was of 27 days duration with a 14 day luteal phase.

LH levels were low in the follicular phase lying at the low end of the normal range. Peak levels on day 0 were just within the normal range, and the peak value persisted for two days. Levels were normal in the luteal phase. FSH levels were within the normal range throughout, but the early follicular, mid-cycle and late luteal phase rises which are associated with the normal cycle did not occur.

FIG. 5. IV COMPARISON OF HORMONE PATTERNS IN INFERTILE WOMEN WITH THE NORMAL MENSTRUAL CYCLE RANGES.



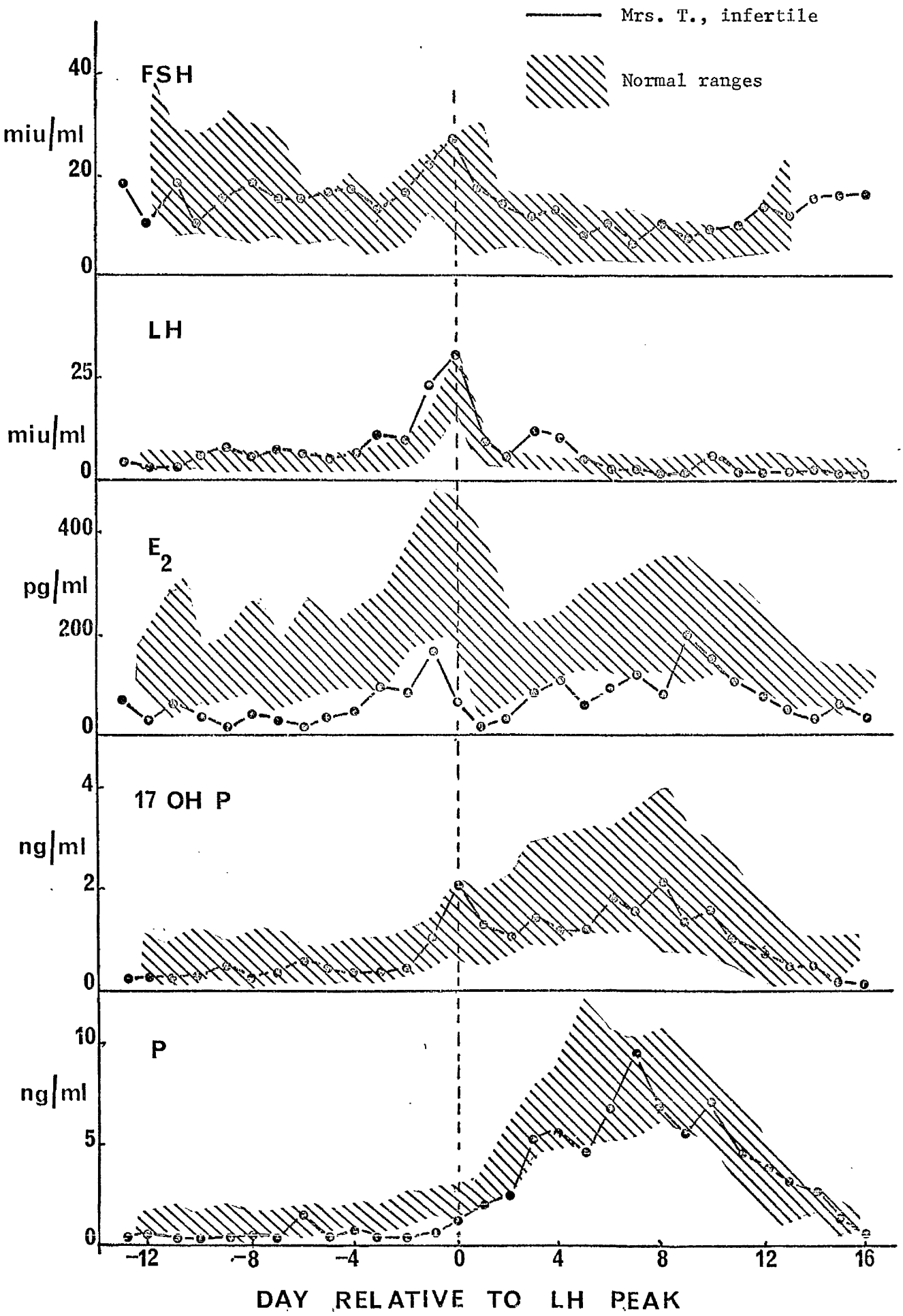
Oestradiol levels were normal in the follicular phase and at the high end of the normal range at mid-cycle. In the luteal phase, the oestradiol levels fluctuated, showing sharp drops on days+4,+8 and+11. Levels were nevertheless within the normal range. 17α-hydroxyprogesterone levels were at the low end of the normal range in the follicular phase, and fell below the normal range from days -4 to 0. Neither a discrete mid-cycle peak nor a significant rise of 17α-hydroxyprogesterone occurred by day 0. Levels fell just within the normal range in the luteal phase. Progesterone levels began above the normal range but fell within that range from day -9 onwards. Luteal phase levels were within the normal range but the pattern showed a slightly retarded rise, peak levels not being attained until day +9.

Mrs. T. - This patient was 31 years old and had been attempting to conceive for 4 years. She had a history of regular cycles of 28 days duration. She had patent fallopian tubes and a secretory endometrium. Biopsy of the ovary on day 21 of her cycle revealed adequate primordial follicles, a developing Graafian follicle, a corpus albicans, which indicated previous ovulation, and a large

atretic Graafian follicle of the type seen in an ovulatory cycle. The hormone levels throughout a cycle were compared to the normal range and are shown in Fig. 5. V. The cycle was of 29 days duration with a 15 day luteal phase.

LH levels were within the normal range for most of the follicular phase and just within the normal range at mid-cycle. In the luteal phase levels were above the normal range from days +2 to +4 but within that range for the remainder of the cycle. FSH levels were normal throughout. Oestradiol levels were very low in the follicular phase and although they increased prior to the LH peak, mid-cycle peak levels were below the normal range. Levels dropped sharply by day +1 and rose in the luteal phase but did not reach the normal range until day +9. 17α-hydroxyprogesterone levels were within the normal range in the follicular phase and displayed a peak on day 0. Luteal phase levels were within the normal range and reached peak values by day +8. Progesterone levels were low in the follicular phase but rose to within the normal range in the luteal phase, reaching peak values by day +7, two days later than in the normal cycle.

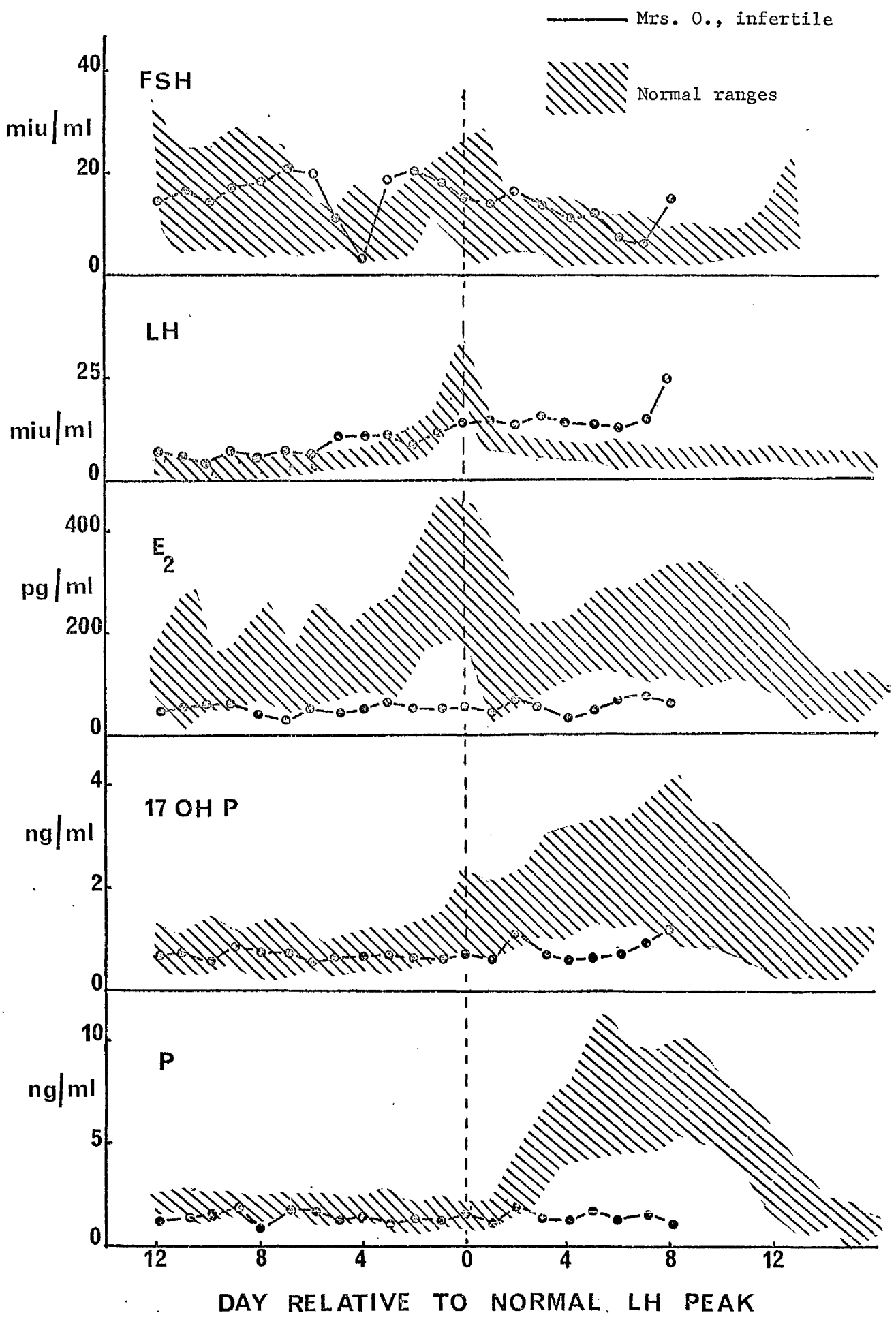
FIG. 5. V COMPARISON OF HORMONE PATTERNS IN INFERTILE WOMEN WITH THE NORMAL MENSTRUAL CYCLE RANGES.



Mrs. O. - This patient was 25 years old, nulliparous and had been attempting to conceive for four years. She had a history of cycles of 28 days with occasional short cycles of 21 days. She had patent fallopain tubes. At laparoscopy a corpus luteum was visible on the right ovary indicating previous recent ovulation. She had a proliferative endometrium on day 13 of her cycle. Hormone patterns throughout a cycle were compared to the normal range and are shown in Fig. 5. VI. The cycle was of 22 days duration. No LH peak was present and hence the days of the cycle are numbered relative to the LH peak of the normal range. LH levels were above the normal range in the first half and in the second half of the cycle and rose steeply on the last day of the cycle. FSH levels dropped sharply on day -4 but were generally within the normal range. Oestradiol levels were below the normal range throughout and showed no fluctuations. 17α-hydroxyprogesterone levels were within the normal range in the first half, showed no mid-cycle rise, and no rise in the second half of the cycle. Progesterone levels were within the normal range in the first half of the cycle but did not rise in the second half.

This cycle lacks the characteristics of a normal cycle and is presumably anovulatory. It therefore differs from the other cycles.

FIG. 5. VI COMPARISON OF HORMONE PATTERNS IN INFERTILE WOMEN WITH THE NORMAL MENSTRUAL CYCLE RANGES.



SUMMARY OF RESULTS IN INFERTILE PATIENTS

From the results obtained in these patients, it appeared that, apart from the anovulatory patient, they could be classified into two groups. Firstly, those who showed low levels of oestradiol in the follicular phase followed by either low progesterone levels in the luteal phase, or levels which took longer to reach peak values than in the normal cycle, (Mrs. T., Fig. 5. V; Mrs. C., Fig. 5. III; Mrs. B., Fig. 5. I), secondly, those who had normal oestradiol levels in the follicular phase and at mid-cycle but still exhibited retarded hormone patterns in the luteal phase (Mrs. R., Fig. 5. II; Mrs. Mc. Fig. 5. IV).

GROUP I

Mrs. T., Mrs. C. and Mrs. B. - In each of these cycles LH peaks were within, or in one case, (Mrs. C.), above the normal range. Oestradiol levels were below the normal range in the follicular phase and showed little or no fluctuation in levels. Mid-cycle peaks occurred (not proven in Mrs. C.) with levels in one case below the normal range (Mrs. T.). The mid-cycle increase began later than in the normal cycle.

17α-hydroxyprogesterone showed normal patterns throughout. Mrs. T, Mrs. B. and Mrs. C. had mid-cycle peaks. Progesterone levels were normal in the follicular phases but in two cases (Mrs. C. and Mrs. B.) levels barely reached the normal range in the luteal phase. In all three cycles progesterone took a longer time to reach peak levels than in the normal cycle.

GROUP 2

Mrs. R. and Mrs. Mc. - In both these cycles the LH pattern was normal throughout. Oestradiol levels were within the normal range in the follicular phase and showed typical patterns, rising to mid-cycle peaks at the high end of the normal range. Levels fluctuated in the luteal phase. Neither cycle had a mid-cycle 17α-hydroxyprogesterone peak nor significant rise before the LH peak. In the luteal phase levels were within the normal range. In both cycles progesterone levels were high on the first day of the cycle, but normal for the remainder of the follicular phase. The luteal rise barely reached normal levels in one case (Mrs. R.) and in both cycles took longer to reach peak levels than the normal cycle.

Further evidence was obtained from these cycles to suggest the peak value of LH was entirely independent of the peak value of oestradiol.

It was interesting to note that these cycles all had normal length luteal phases.

The area under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phase was related to luteal peak value of the steroids (Table 5. 1). There was good correlation between peak height and area; in most cases, the greater the peak height the greater the area under the curve. However, there was no correlation between peak height or area of one hormone and that of another hormone in the same cycle.

THE MEAN CYCLE

As the cycles showed many individual features, it may be invalid to compare the mean levels in the infertile series with the normal mean levels. However, when this was done, an overall picture of the differences between the infertile and normal groups was obtained (the results for Mrs. O. were omitted from calculations of mean levels since this cycle was apparently anovulatory).

TABLE 5. 1. COMPARISON OF LUTEAL PEAK LEVELS OF PROGESTERONE, 17α-HYDROXYPROGESTERONE, AND OESTRADIOL WITH AREA UNDER THEIR CURVES IN THE LUTEAL PHASES OF MENSTRUAL CYCLES FROM INFERTILE SUBJECTS.

SUBJECT	LUTEAL PHASE (DAYS)	PROGESTERONE (ng)		17α-HYDROXYPROGESTERONE (ng)		OESTRADIOL (pg)	
		AREA	PEAK HEIGHT	AREA	PEAK HEIGHT	AREA	PEAK HEIGHT
Mrs. R.	14	55.4	6.5	20.9	2.3	2670	245
Mrs. Mc.	14	78.2	9.0	14.6	1.7	2395	290
Mrs. C.	15	49.2	5.9	27.1	2.7	1330	160
Mrs. B.	16	68.3	6.9	22.6	2.6	1710	155
Mrs. T.	16	71.8	9.3	17.9	2.1	1195	200

The mean LH levels ± S.D. (n = 5) were compared to the normal range and are shown in Fig. 5. VII. The infertile range was higher than the normal range in the follicular phase and the mean infertile level at mid-cycle was slightly higher than the normal mean level. In the luteal phase, the ranges were similar.

The mean FSH levels ± S.D. (n = 5) were compared to the normal range and are shown in Fig. 5. VIII. Levels tended to be lower than the normal mean in the early follicular phase (days -12 to -9). Levels at mid-cycle were similar to the normal range, and the luteal phase pattern closely followed the normal cycle.

The mean oestradiol levels ± S.D. (n = 5) were compared to the normal range and are shown in Fig. 5. IX. In the follicular phase, the infertile mean was lower than the normal mean and did not show such marked fluctuations in levels. Mid-cycle peak ranges were not significantly different. In the luteal phase the infertile mean was below the normal mean (from days +2 to +8) and comparison of the two ranges showed that oestradiol levels in the normal cycle peaked 3 days before those in the infertile cycle.

The mean 17α-hydroxyprogesterone levels ± S.D. (n = 5) were compared to the normal range and are shown in Fig. 5. X.

FIG. 5. VII THE MEAN LEVELS OF LH IN INFERTILE WOMEN ± S.D. (n = 5) COMPARED TO THE NORMAL RANGE

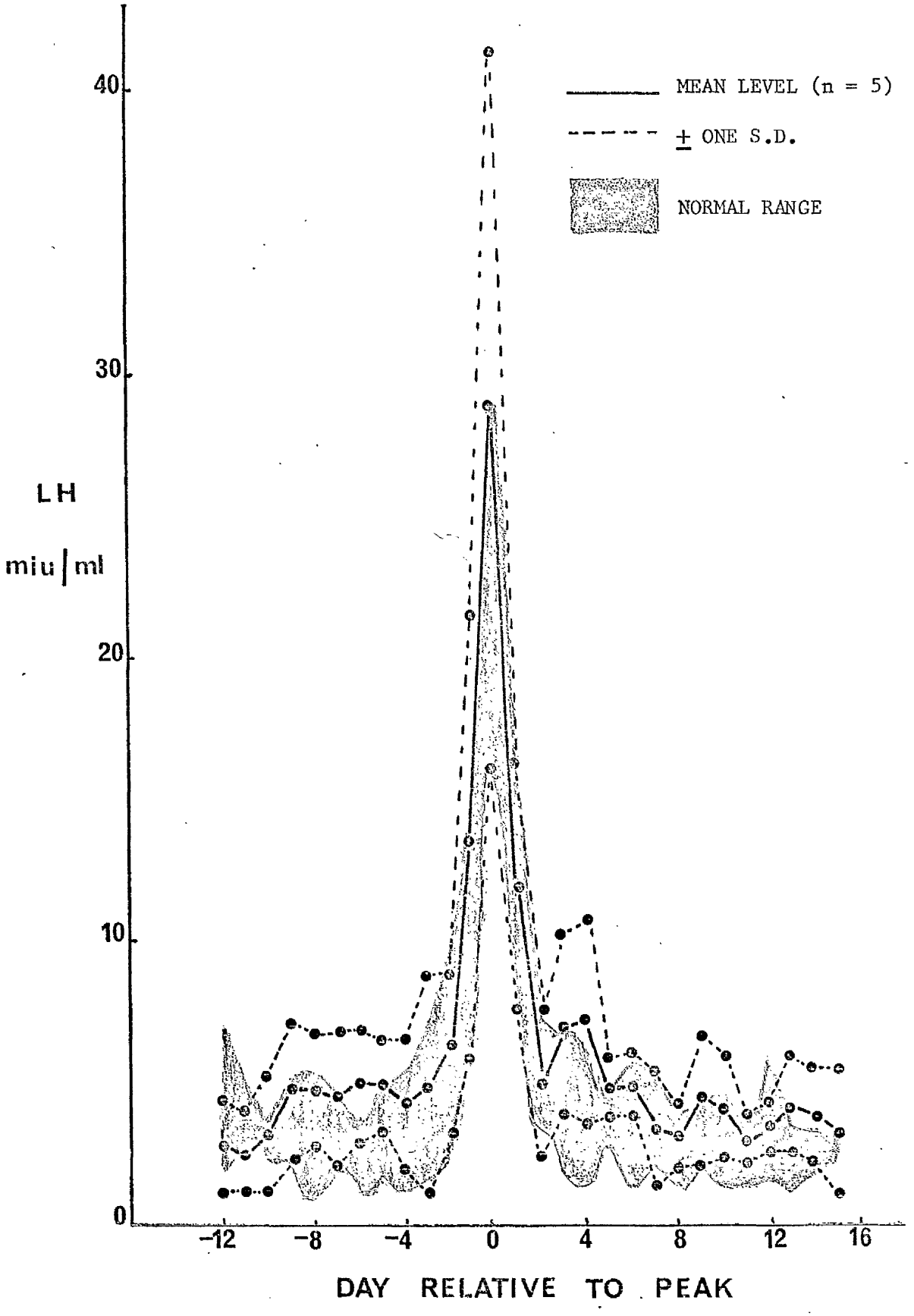


FIG. 5. VIII THE MEAN LEVELS OF FSH IN INFERTILE WOMEN ± S.D. (n = 4) COMPARED TO THE NORMAL RANGE

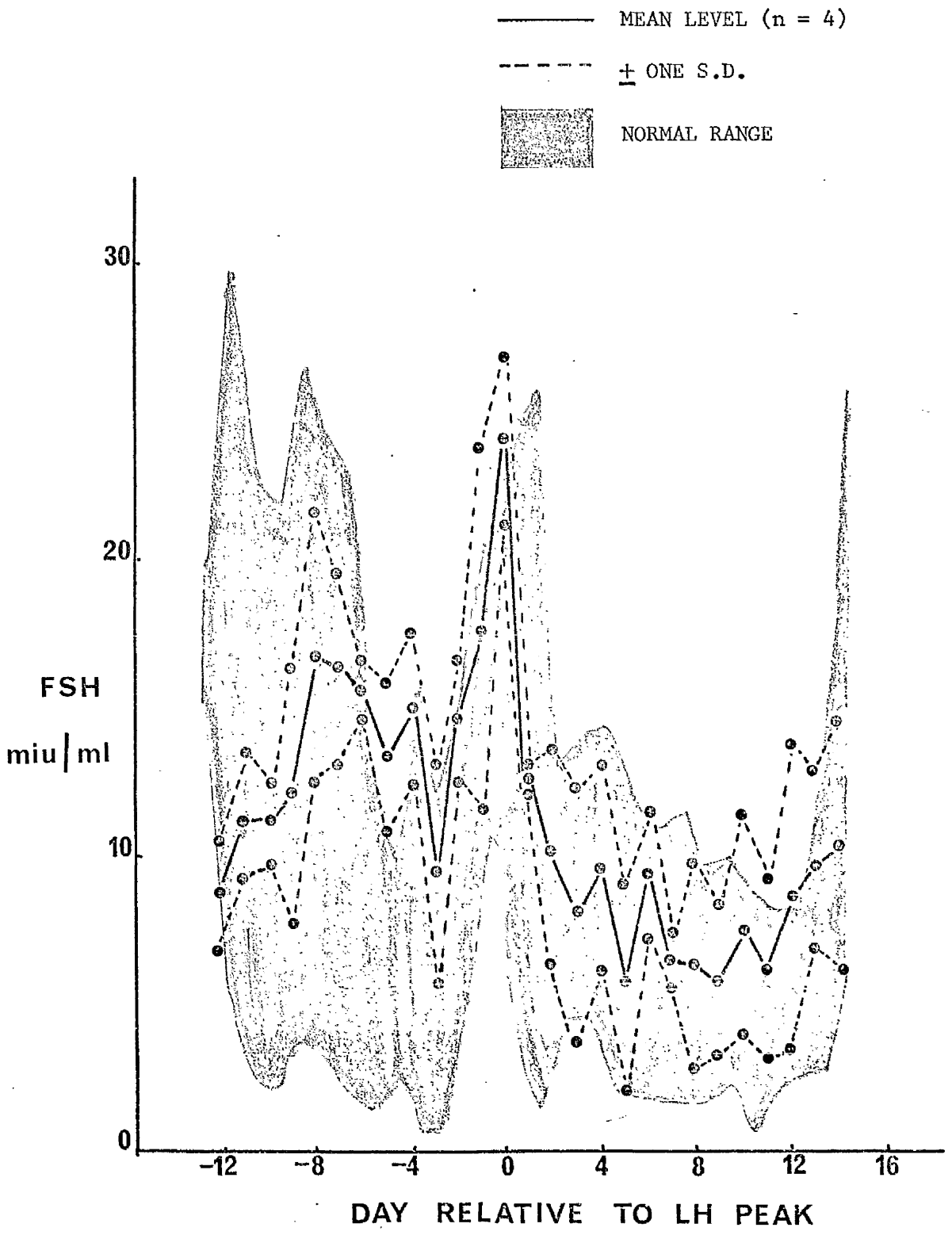


FIG. 5. IX THE MEAN LEVEL OF OESTRADIOL (E_2) IN INFERTILE WOMEN ± S.D. (n = 5) COMPARED TO THE NORMAL RANGE

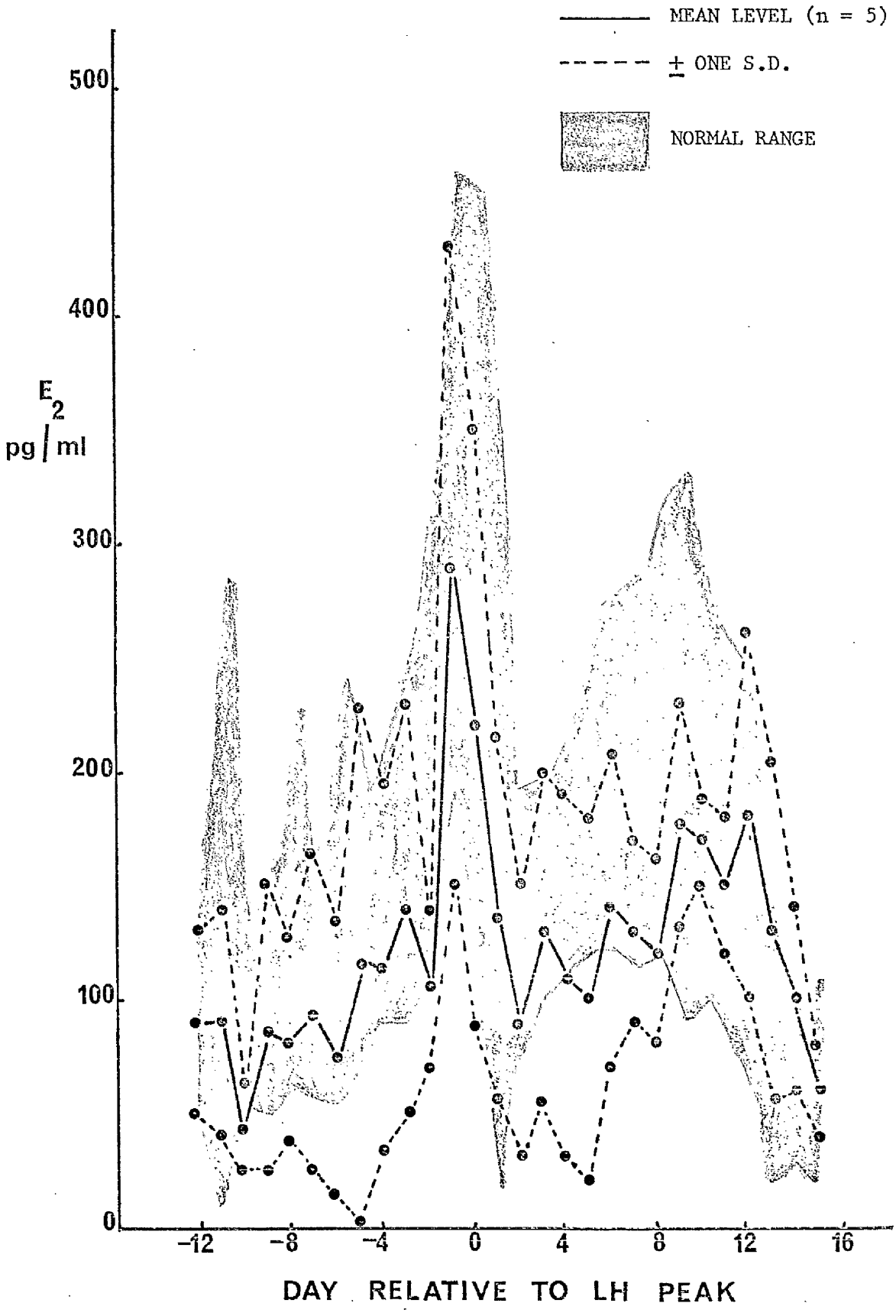


FIG. 5. X THE MEAN LEVEL OF 17∝-HYDROXYPROGESTERONE (17 OHP) IN INFERTILE WOMEN ± S.D. (n = 5) COMPARED TO THE NORMAL RANGE.

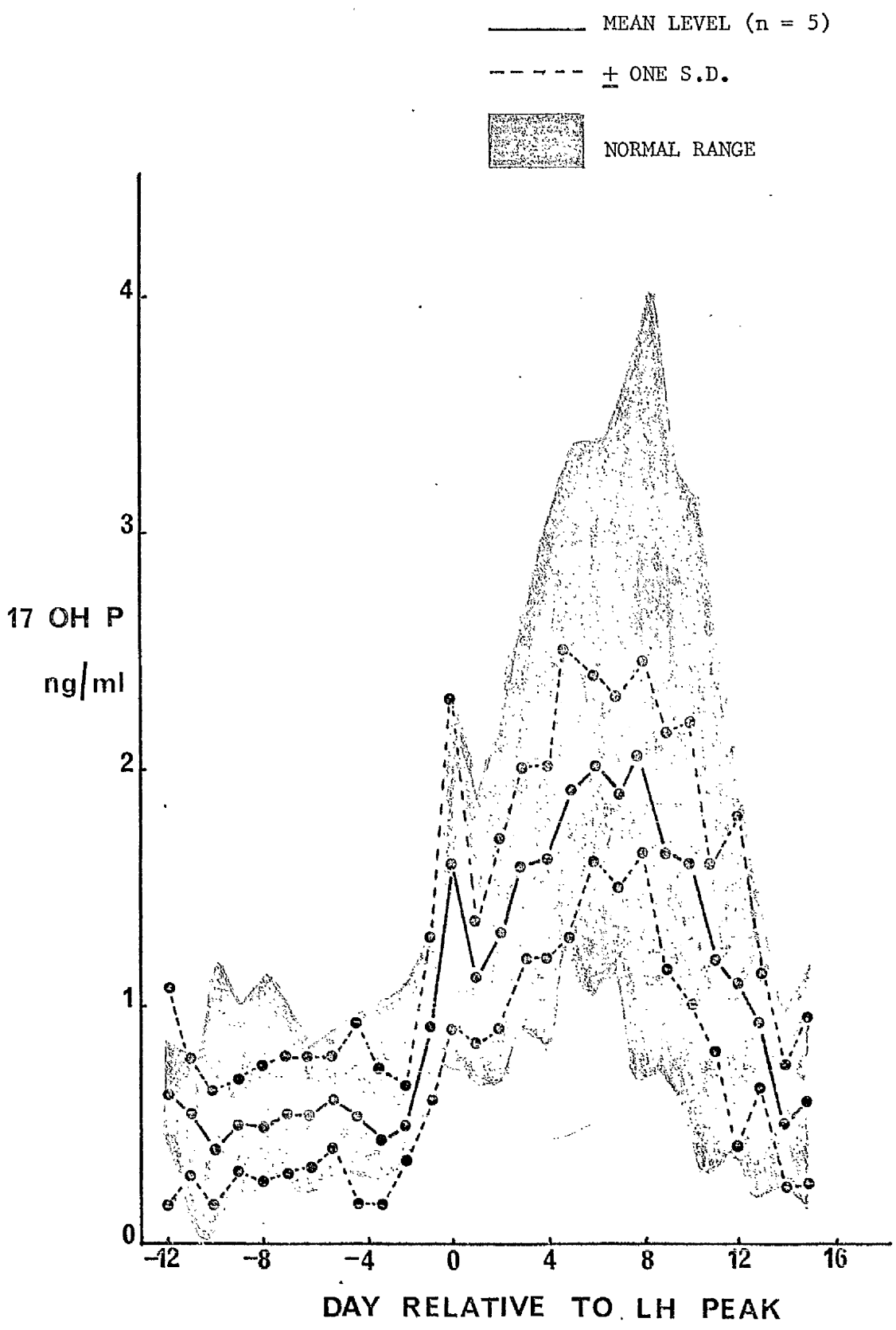
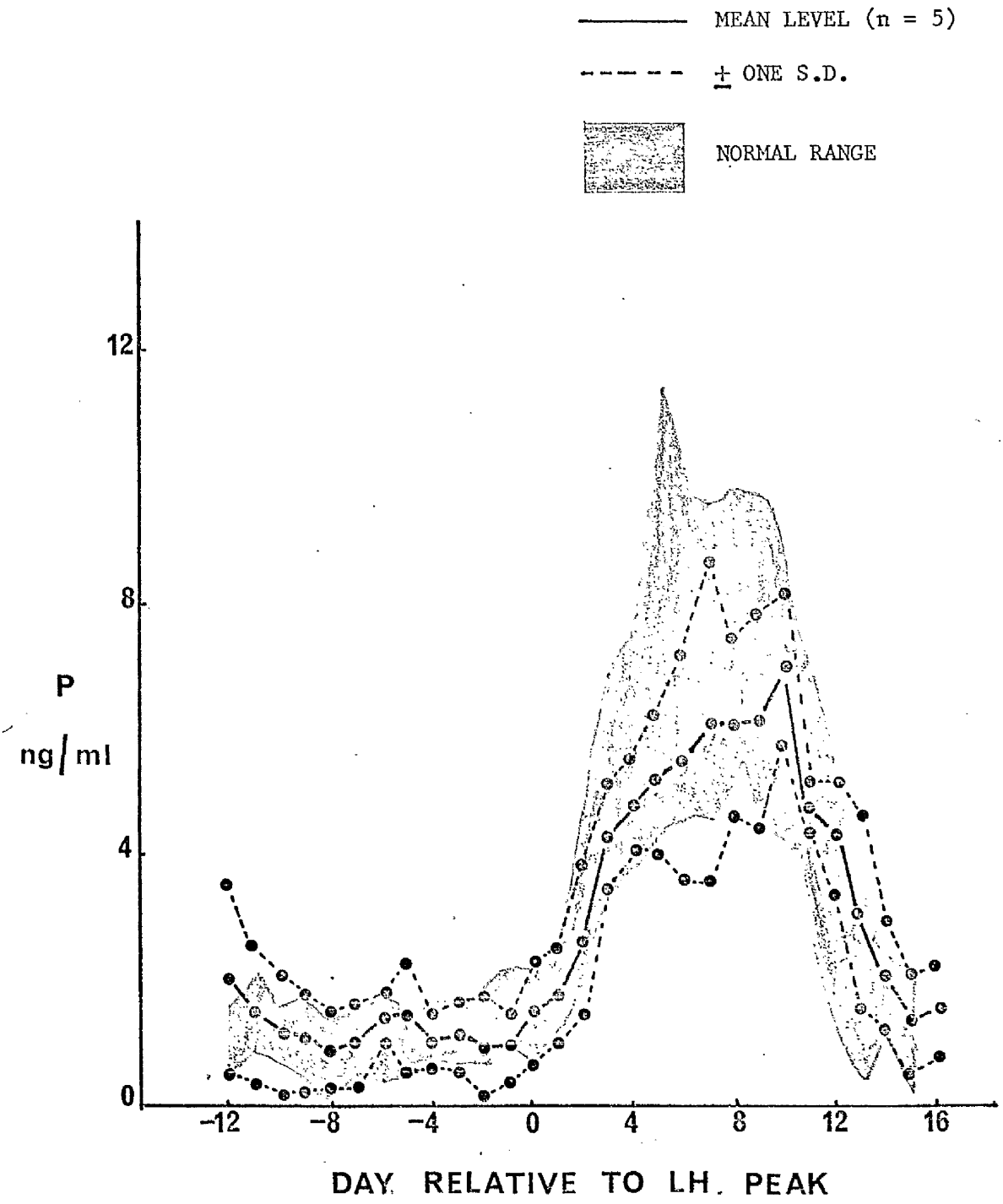


FIG. 5. XI THE MEAN LEVEL OF PROGESTERONE (P) IN INFERTILE WOMEN ± S.D. (n = 5) COMPARED TO THE NORMAL RANGE.



The ranges were not significantly different at any point and showed similar patterns throughout.

The mean progesterone levels $\pm$ S.D. (n = 5) were compared to the normal range and are shown in Fig. 5. XI. The ranges were similar in the follicular phase. In the luteal phase, the infertile range was slightly below the normal range. However, the most marked difference in the two patterns was the rate of progesterone increase in the luteal phase. The progesterone normal range rose more steeply and reached peak values several days before that of the infertile range.

CHAPTER 6

HORMONE PATTERNS IN THE MENSTRUAL CYCLES OF INFERTILE WOMEN AFTER TREATMENT WITH 'CLOMID'

HORMONE PATTERNS IN THE MENSTRUAL CYCLES OF INFERTILE WOMEN AFTER TREATMENT WITH 'CLOMID'

INTRODUCTION

The results presented in the previous chapter indicated that whilst most of the normal hormonal events occurred in the menstrual cycles of these infertile women, subtle differences did exist between levels and patterns of hormones in the infertile series and those in the normal series. Five patients who fulfilled the criteria desired in the study were apparently ovulatory and one was not. However, hormone patterns in the follicular and luteal phases suggested abnormalities in ovarian function. FSH tended to be lower in the early follicular phase in infertile women and follicular phase levels of oestradiol were low in some cases. In spite of this, in five women LH was released and a corpus luteum was apparently produced with a retarded rate of hormone production. It must be emphasised however, that these cycles did not all show the same abnormalities. The patterns were very individual which suggested that the reason for infertility was not necessarily the same in each subject.

There are two main therapeutic methods of treating anovulatory infertility; by injection of human pituitary or menopausal

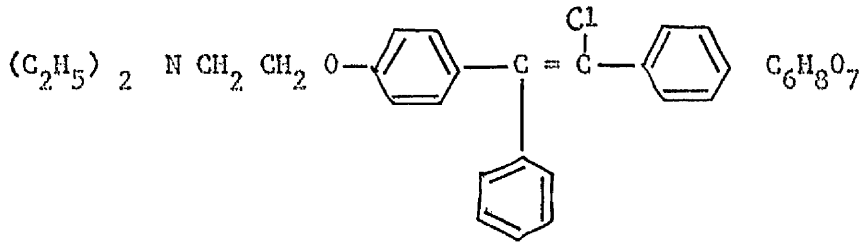
gonadotrophin, 'Pergonal', followed by chorionic gonadotrophin or by oral administration of 'Clomid'. Both of these preparations have previously been used in anovulatory or oligo-ovulatory conditions. 'Pergonal' is used in cases of pituitary failure, as a substitute for endogenous gonadotrophins, and treatment must be carefully monitored on a daily basis to prevent hyper-stimulation and the possibility of a multiple pregnancy. 'Clomid' on the other hand, is a synthetic compound used when the pituitary is capable of producing gonadotrophins on stimulation. 'Pergonal' was unlikely to be useful in the treatment of these patients as their pituitary function was apparently normal. There was evidence however, to suggest that 'Clomid' would help to produce normal ovarian function.

Clomiphene citrate is chemically :-

2- [p - (2 - chloro - 1, 2 - diphenyl vinyl) phenoxy] triethylamine dihydrogen citrate and its structure is shown in Fig. 6. I.

FIG. 6. I.

The structure of clomiphene citrate -



Initial experiments in rats showed the drug to be an inhibitor of gonadotrophin secretion and to have direct blastotoxic effects in fertilised ovum (Holtkamp et al., 1960). This suggested that it might be employed as an oral contraceptive. However, when administered to women, it was found to induce, rather than prevent ovulation.

Its mode of action is not clear (Loraine and Bell, 1968; Papanicoloau et al., 1970). There is some indication that the site of action of the drug is at the hypothalamo-pituitary level and that it acts to increase gonadotrophin secretion (Jacobson et al., 1968; Ross et al., 1970). The effects which 'Clomid' has on hormone levels in urine and plasma have

been shown to vary markedly and other investigators provide evidence to suggest that the compound has a direct action on the ovary (Smith 1966; Papanicoloau et al., 1970). Urinary oestrogen excretion increases consistently following 'Clomid' therapy (Dickey et al., 1965) and levels often exceed those found in the normal cycle, this being observed within 48 hours after beginning treatment (Smith et al., 1963). Urinary excretion of pregnanediol is sometimes greater following ovulation induced by 'Clomid' than during the luteal phase of the normal cycle, and improvement in patients with an inadequate luteal phase has been reported (Roy et al., 1963). Herrmann (1963) was able to produce further increases in pregnanediol excretion by starting administration after ovulation had already occurred. It appears therefore that 'Clomid' may stimulate corpus luteum function as well as induce ovulation.

'Clomid' may be useful in the treatment of these patients, to increase follicular growth and subsequently to improve corpus luteum function. Ovulation may be induced by 'Clomid' therapy in the anovulatory patient (Mrs. O). Treatment from days 1 to 5 in each cycle may increase the early follicular phase levels of FSH and/or early follicular growth.

The six women who had previously been studied returned at the beginning of their next but one cycle. 'Clomid' was

administered orally from days 1 to 5 in four patients (Mrs. B., Mrs. Mc., Mrs. T., and Mrs. O.) and days 6 to 10 in two patients (Mrs. R., and Mrs. C.). Blood samples were collected daily as before and levels of progesterone, 17α-hydroxyprogesterone, oestradiol, LH and FSH were estimated as described previously. The results for these cycles were divided into two groups on the basis of the pre-treatment results as described in the previous chapter (p. 106). The anovulatory patient was considered separately.

RESULTS

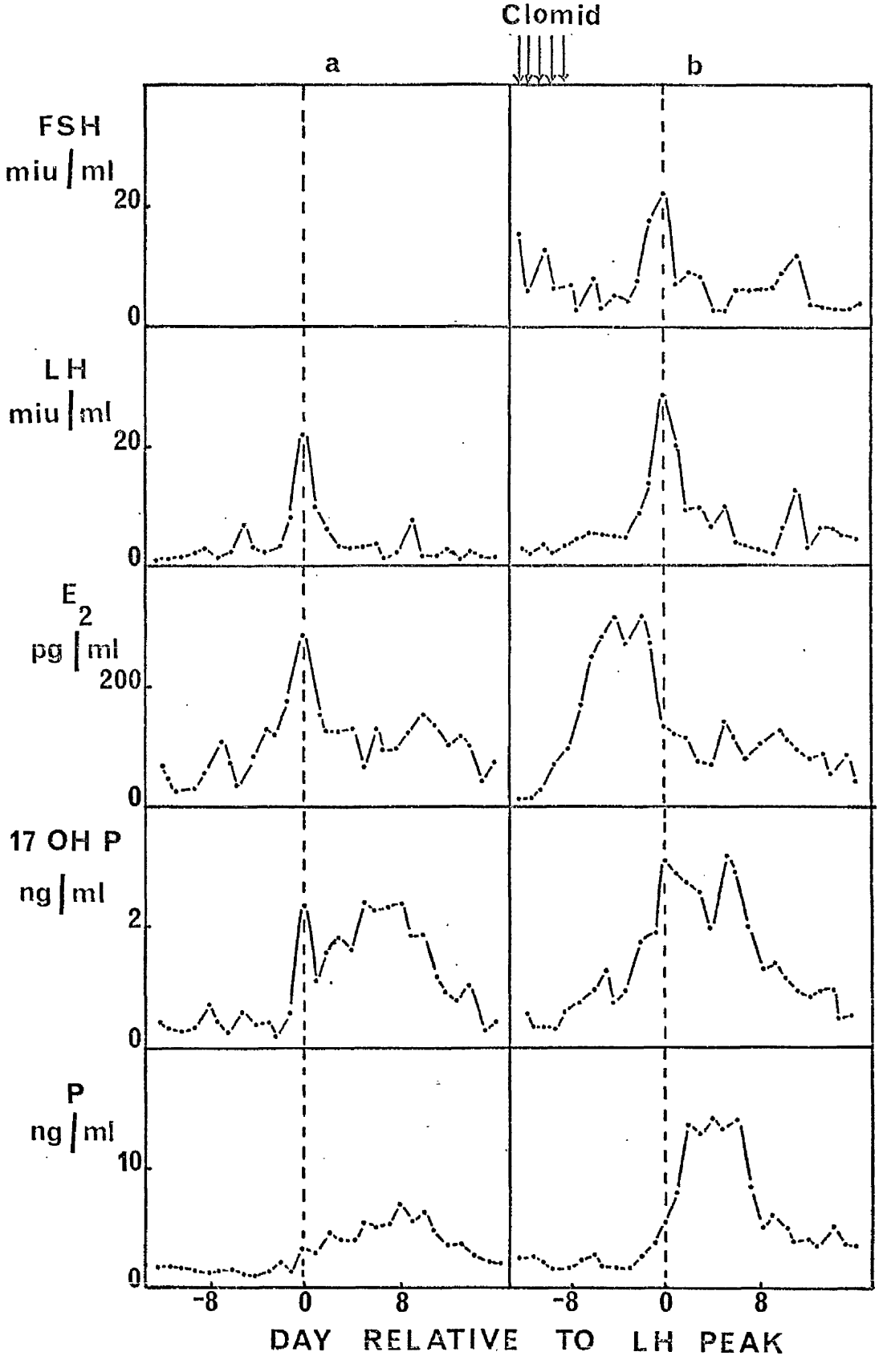
GROUP I

Mrs. B. - The hormone levels before and after treatment are shown in Fig. 6. II. Pre-treatment FSH results were not available. After treatment FSH levels lay within the normal range but lacked the early follicular phase and late luteal phase elevations seen in the normal cycle. Levels of LH at mid-cycle were higher after treatment than before treatment. However, the most marked effects were on the steroid hormone levels, especially oestradiol and progesterone. In the treatment cycle, oestradiol levels began to rise consistently from the fourth day of treatment

FIG. 6. II PLASMA HORMONE LEVELS IN MENSTRUAL CYCLES FROM INFERTILE WOMEN.

Mrs. B. (a) Before treatment
(b) During treatment with 'Clomid' (50mg/day)

Treatment days are indicated by vertical arrows in this and subsequent diagrams.

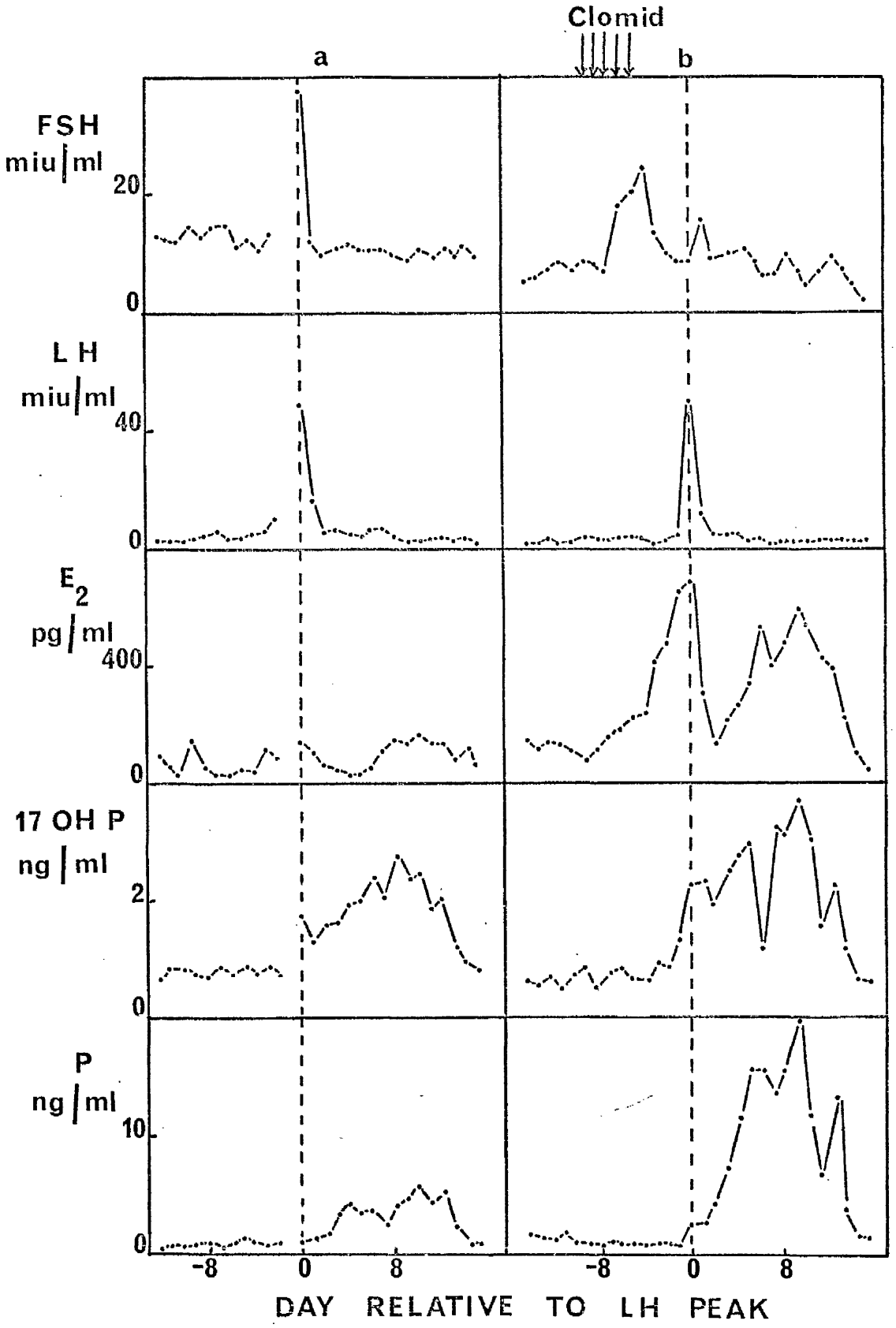


reaching peak values between days -4 and -1. The peak value was no higher than in the pre-treatment cycle but was maintained for a longer period. Luteal phase levels of oestradiol were not altered after treatment although peak values were reached several days earlier. 17α-hydroxyprogesterone levels began to rise from day -2 onwards and this elevation was continued into the luteal phase. Levels were slightly increased in the treatment cycle both at mid-cycle and in the luteal phase. Progesterone levels rose on the day of the LH peak and the rise was sustained into the luteal phase. Luteal phase values were greatly increased after treatment; peak values were reached on day +2 and were maintained for 5 days. However, levels dropped sharply on day+7.

Mrs. C. - The hormone levels before and after treatment are shown in Fig. 6. III. This patient was treated from days 6 to 10 of her cycle. There was a rise in FSH on the fourth day of treatment and a peak was reached on the day following the last treatment day. The mid-cycle FSH peak (day +1) however, was lower than the pre-treatment mid-cycle peak. There was no difference in

FIG. 6. III PLASMA HORMONE LEVELS IN MENSTRUAL CYCLES FROM INFERTILE WOMEN.

Mrs. C. (a) Before treatment
(b) During treatment with 'Clomid' (50mg/day)

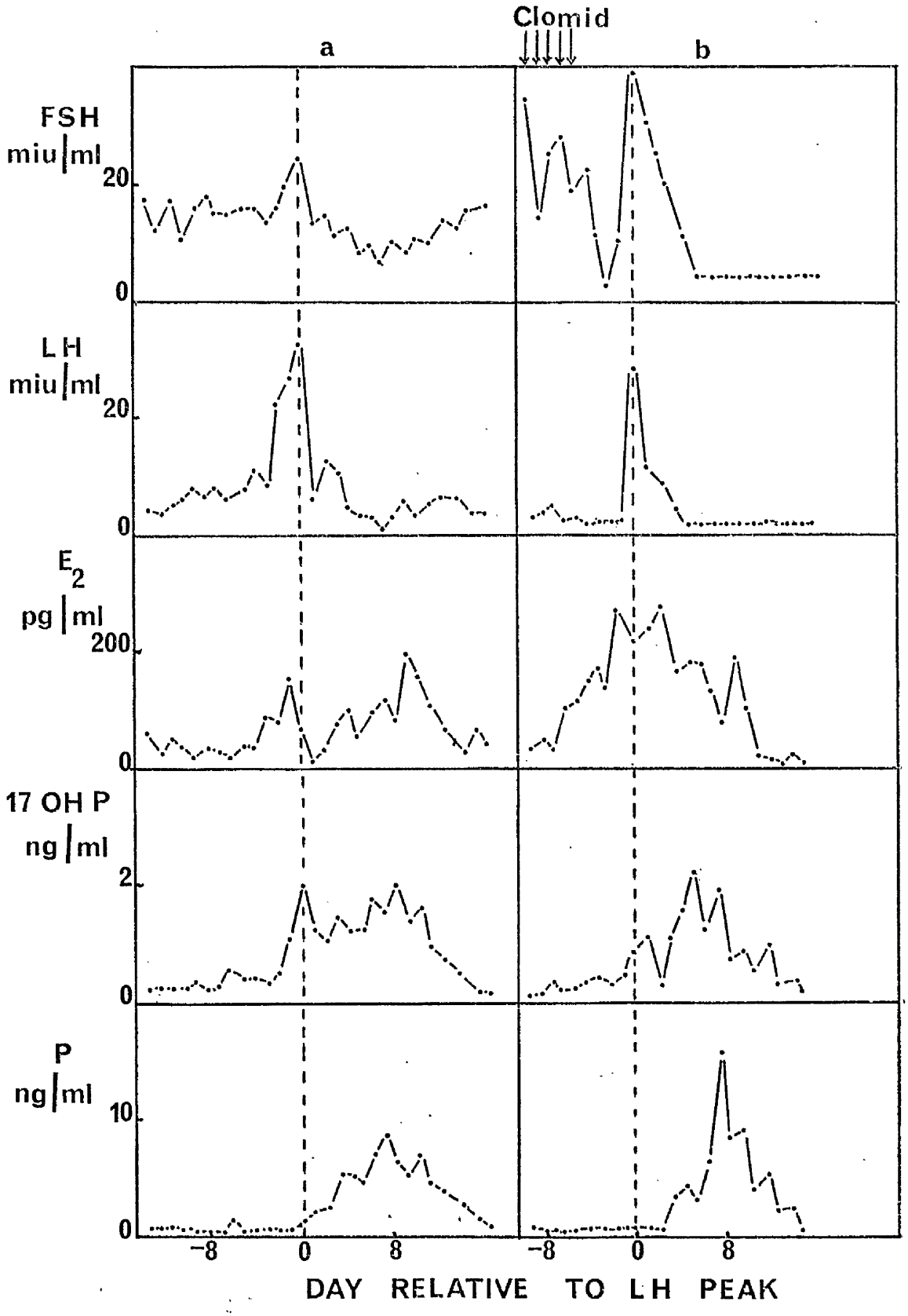


pattern or mid-cycle level of LH before and after treatment. Oestradiol levels began to rise from the second treatment day, reaching peak values (550 pg/ml) on day -1. Unfortunately, the pre-treatment sample for this day was not available but in view of the level on the preceding day it seemed likely that the corresponding (mid-cycle) value in the pre-treatment cycle would have been considerably lower. Luteal phase levels of oestradiol were elevated above pre-treatment levels and reached a peak on day +6 which was above the normal range. 17α-hydroxyprogesterone was not altered in the follicular phase after treatment. However, the mid-cycle and luteal phase levels were elevated. A sharp drop occurred on day +6 in the luteal phase but peak levels were regained by day +7. A rise in progesterone levels was observed on day 0 which continued into the luteal phase. Levels in the luteal phase were greatly elevated after treatment reaching a peak value of 20 ng/ml which was well above the normal range. Levels above the normal range were reached by day +5. The luteal phase patterns of progesterone and 17α-hydroxyprogesterone were similar in both the pre-treatment and the 'Clomid' treatment cycles.

Mrs. T. - The hormone levels before and after treatment are shown in Fig. 6. IV. FSH levels in the early follicular phase were generally higher in the treatment cycle than in the pre-treatment cycle. However, the higher value on day 1 of treatment could not have been a result of treatment since this sample was collected immediately before the first dose of 'Clomid' was administered. Mid-cycle levels of FSH were also elevated in the treatment cycle, whilst the normal late luteal phase rise was absent. The level of LH at mid-cycle was not significantly altered in the treatment cycle. Oestradiol levels began to rise consistently from the fourth day of treatment onwards, reaching a peak on day -1, which was higher than in the pre-treatment cycle. This peak value was maintained into the luteal phase. The drop which normally occurred after the LH peak was absent. Peak luteal phase levels were maintained until day +3 when they began to decrease. Luteal phase levels, in general, were slightly higher than in the pre-treatment cycle. 17α-hydroxyprogesterone levels were lower, at mid-cycle, in the treatment cycle than in the pre-treatment cycle. Luteal peak levels, however, were reached earlier (day +5) in the treatment cycle, although the levels were similar

FIG. 6. IV PLASMA HORMONE LEVELS IN MENSTRUAL CYCLES FROM INFERTILE WOMEN.

Mrs. T. (a)Before treatment
(b)During treatment with 'Clomid' (50 mg/day)



in both cycles. Luteal phase peak progesterone levels were elevated in the treatment cycle, although a level above the normal range was maintained for only one day. This peak level was not reached until day +7, as in the pre-treatment cycle.

GROUP 2

Mrs. R. - The hormone levels before and after treatment are shown in Fig. 6. V. Comparison of the pre-treatment results with the results after treatment with 50 mg of 'Clomid' from days 6 to 10 indicates that in this patient levels of each hormone were depressed following 'Clomid' treatment. Blood samples were not collected until the last treatment day. FSH results were not available for this treatment cycle. LH levels at mid-cycle were lower after treatment. Oestradiol mid-cycle levels were lower and 17α-hydroxyprogesterone levels were lower in the luteal phase. Because of this lack of response to treatment, the dose was increased to 100 mg per day from days 5 to 9, of the following cycle. The results are shown in Fig. 6. V (c). FSH levels in the early follicular phase were higher than in the pre-treatment cycle, although this elevation was apparent before treatment began. Mid-cycle and luteal

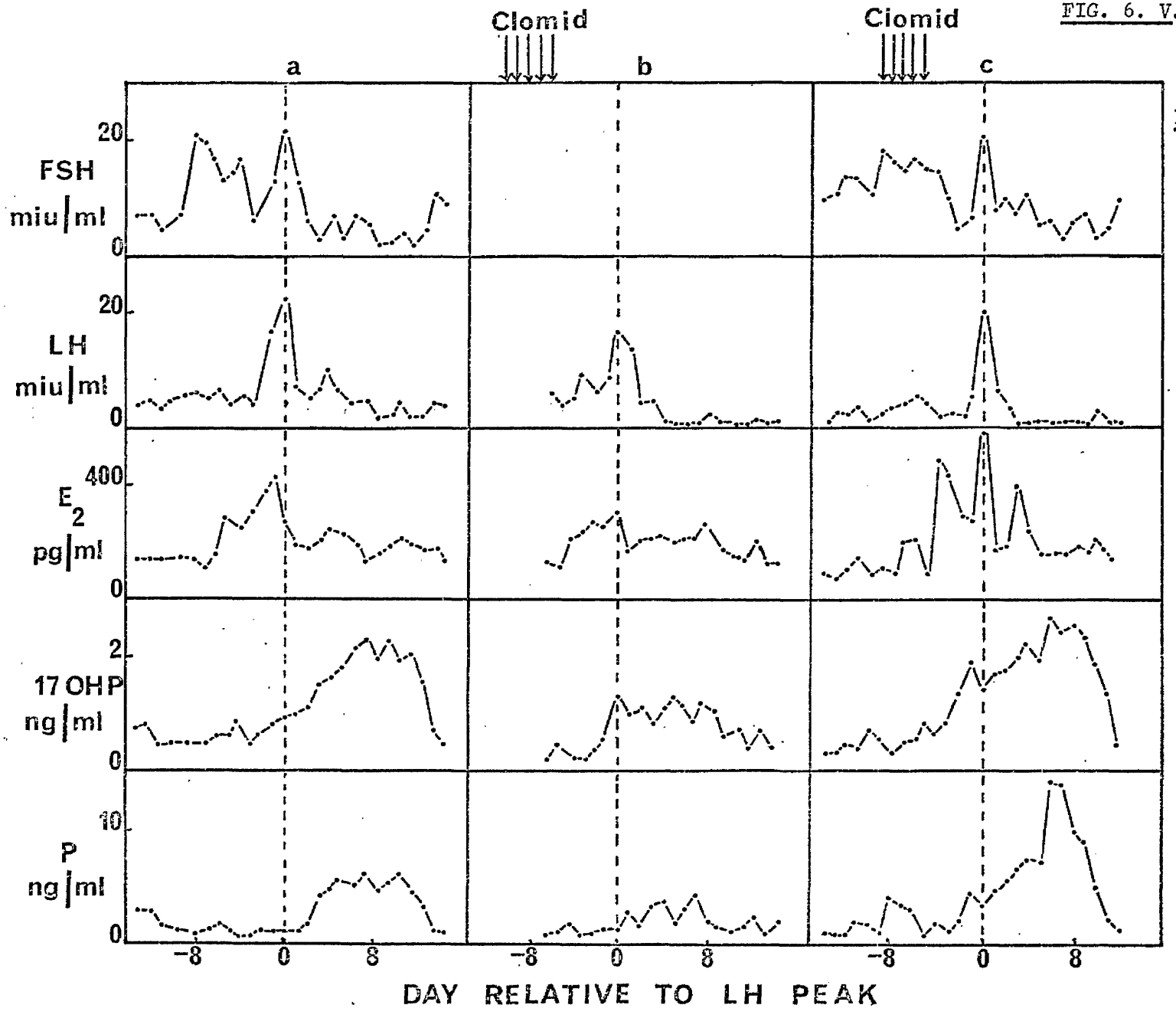


FIG. 6. V. PLASMA HORMONE LEVELS IN MENSTRU
CYCLES FROM INFERTILE WOMEN

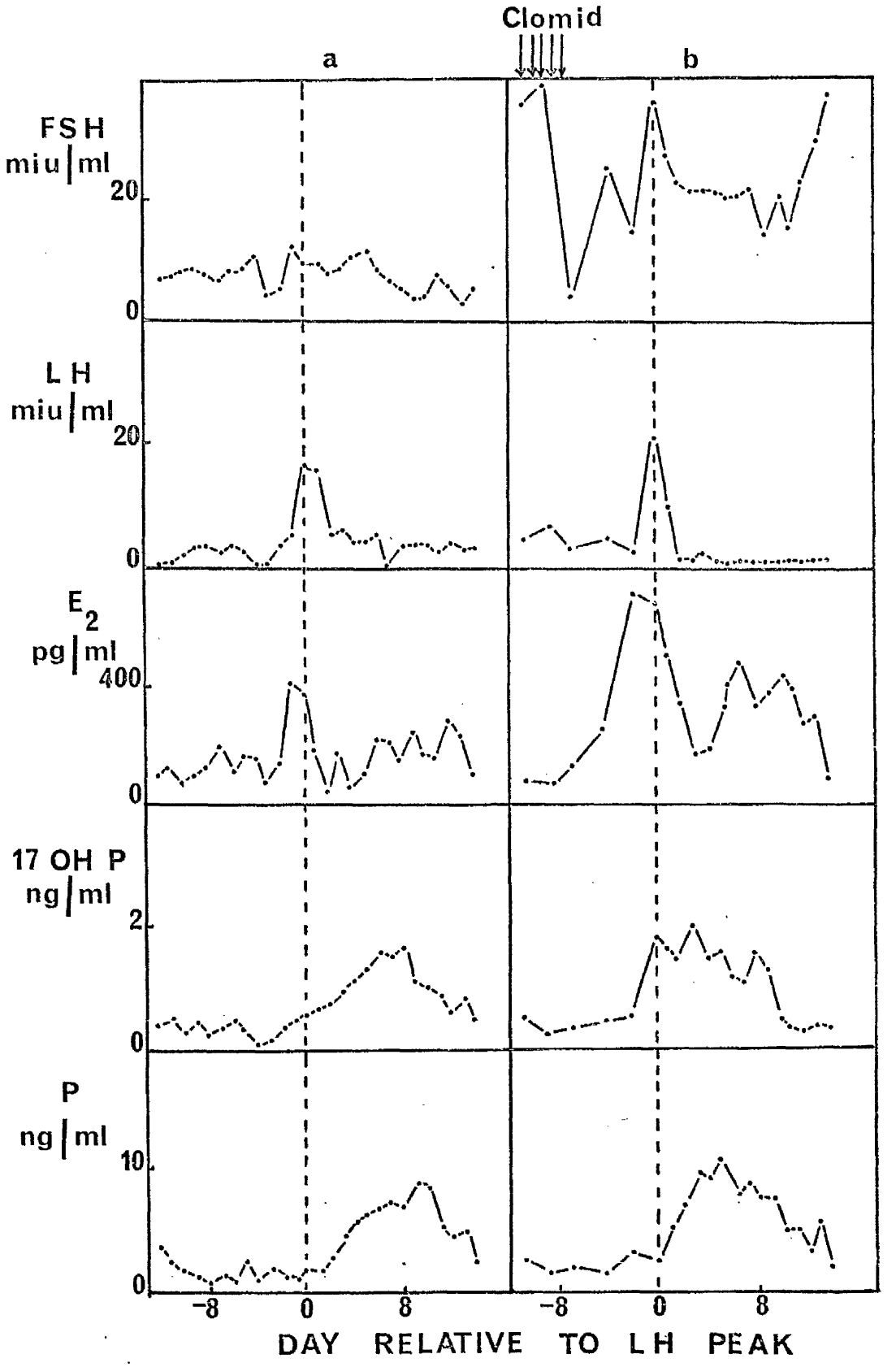
Mrs. R. (a) Before treatment
(b) During treatment with 'Clomid' (50mg/day)
(c) During treatment with 'Clomid' (100 mg/day)

phase levels of FSH were unaltered from the pre-treatment cycle. LH was restored to the original, pre-treatment mid-cycle level. A rise in oestradiol occurred after the final day of treatment and a mid-cycle peak, of greater magnitude than in the pre-treatment cycle was observed on day 0. A luteal phase peak of oestradiol occurred on day +3, which was higher than in the pre-treatment cycle, but this peak level was not maintained. The 17α-hydroxy-progesterone level at mid-cycle was elevated but the luteal phase levels were similar to those of the pre-treatment cycle. Progesterone levels rose above normal levels in the follicular phase on the third day of treatment and showed a significant rise one day before the LH peak. Luteal phase levels were elevated above pre-treatment levels and rose above the normal range by day +6. Progesterone levels dropped steadily from day +7 to the end of the cycle.

Mrs. Mc.- The hormone levels during cycles before and after treatment with 'Clomid' from days 1 to 5 are shown in Fig. 6. VI. Unfortunately, the patient only attended on alternate days to have blood samples removed during the first half of her treatment cycle.

FIG. 6. VI PLASMA HORMONE LEVELS IN MENSTRUAL CYCLES FROM INFERTILE WOMEN.

Mrs. Mc (a) Before treatment
(b) During treatment with 'Clomid' (50 mg/day)



FSH levels in the early follicular phase of the treatment cycle were higher than in the pre-treatment cycle. However, this elevation was not due to treatment as the level was high in the sample taken before the first dose of 'Clomid' was administered. A sharp drop in FSH levels occurred by the final day of treatment and levels again rose reaching a mid-cycle peak, which was higher than in the pre-treatment cycle. Levels dropped, as in the normal cycle, in the early luteal phase and then rose in the late luteal phase. The pattern and mid-cycle level of LH was similar in both treatment and pre-treatement cycles. Oestradiol levels, conversely, were vastly elevated in the treatment cycle. The mid-cycle rise began after the last day of treatment and culminated in a peak above the normal range falling between days -2 and 0. Luteal phase levels of oestradiol were also higher in the treatment cycle. 17α-hydroxyprogesterone showed a mid-cycle rise in the treatment cycle which was not apparent in the pre-treatment cycle. Luteal phase levels of 17α-hydroxyprogesterone were similar in the two cycles. Progesterone levels rose significantly two days before the LH peak in the treatment cycle. The luteal phase peak levels, although of similar magnitude in the two cycles, were

reached on day +5 of the treatment cycle, compared to day +9 of the pre-treatment cycle.

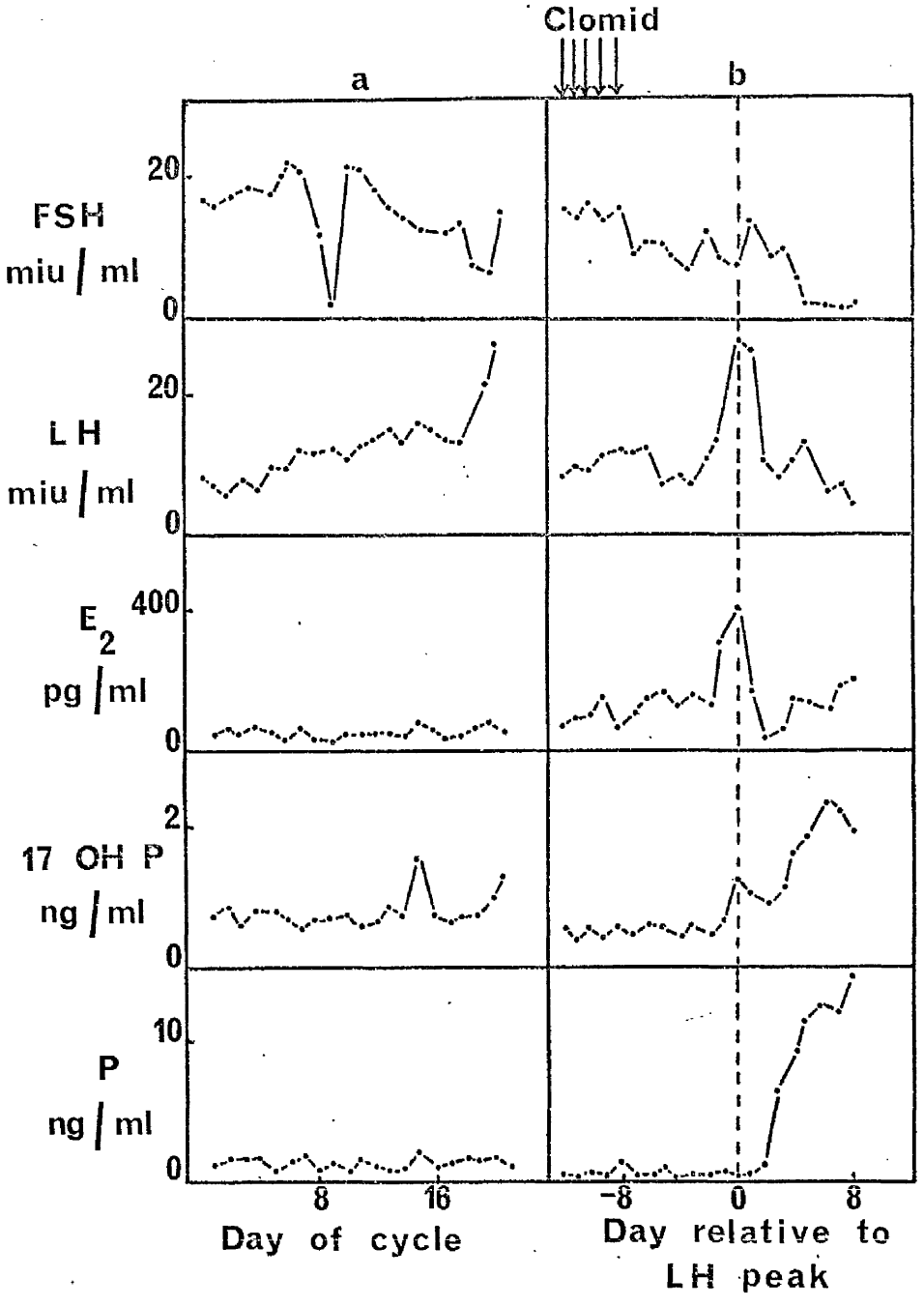
ANOVULATORY PATIENT

Mrs. O. - The hormone levels before and after treatment are shown in Fig. 6. VII. This patient, the only one who was apparently anovulatory, became pregnant on treatment with 'Clomid'. Blood samples were collected for only 21 days of the treatment cycle.

FSH levels, in the treatment cycle, were higher in the early follicular phase than at mid-cycle; the mid-cycle peak occurred on day +1. In the treatment cycle, a mid-cycle peak of LH was present. Oestradiol levels rose by the fourth day of treatment. They reached a mid-cycle peak within the normal range on the day of the LH peak. 17α-hydroxyprogesterone levels were not altered in the follicular phase of the treatment cycle. Levels rose on the day of the LH peak and the rise was continued into the luteal phase. Progesterone levels rose progressively from day +2 onwards. Both progesterone and 17α-hydroxyprogesterone reached normal range luteal peak levels by 4 days after the LH peak. The pregnancy has so far been clinically uneventful.

FIG. 6. VII PLASMA HORMONE LEVELS IN MENSTRUAL CYCLES FROM INFERTILE WOMEN.

Mrs. O. (a) Before treatment
(b) During treatment with 'Clomid' (50 mg/day)



The areas under the progesterone, 17α-hydroxyprogesterone and oestradiol curves in the luteal phases of the treatment and pre-treatment cycles are compared in Table 6.1. The luteal phase peak heights of progesterone, 17α-hydroxyprogesterone and oestradiol in the treatment and pre-treatment cycles are compared in Table 6.2.

THE MEAN TREATMENT CYCLE AND COMPARISON WITH THE NORMAL CYCLE

The patients varied in their response to 'Clomid' but nevertheless comparison of the mean treatment levels with the normal levels gave an indication of the general effects of treatment.

In calculating mean levels, all treatment cycles were included except that of Mrs. R. (cycle 2).

The mean LH levels ± S.D. (n = 6) were compared to the normal range and are shown in Fig. 6. VIII. The treatment range was similar, although wider than the normal range in the follicular phase. The mean level at mid-cycle was higher in the treatment cycle than in the normal cycle. The ranges were similar in the luteal phase.

The mean FSH levels ± S.D. (n = 6) were compared to the

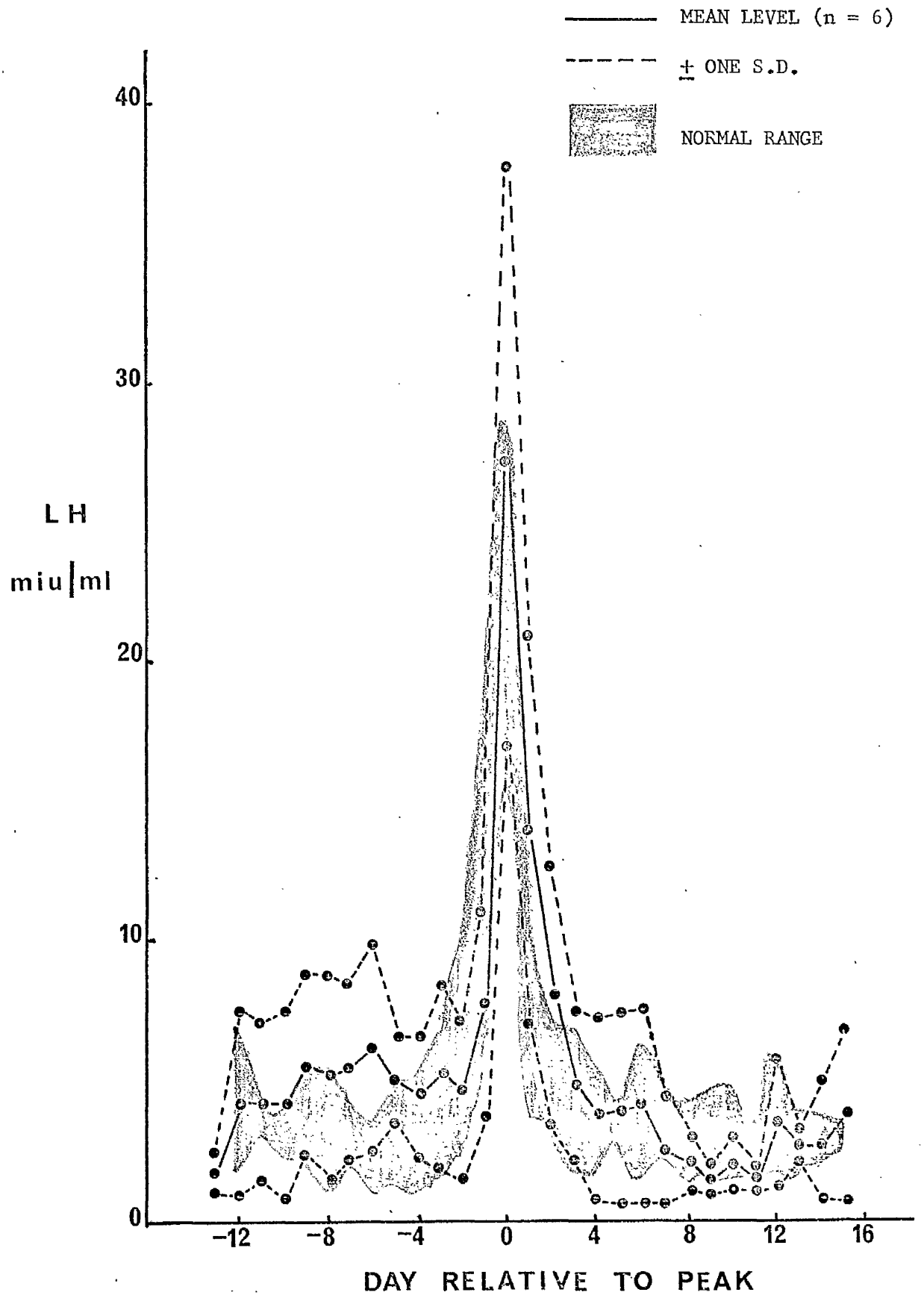
TABLE 6. 1. COMPARISON OF AREAS UNDER THE LUTEAL PHASE CURVES OF PROGESTERONE (PROG.) 17α-HYDROXYPROGESTERONE (17 OHP) AND OESTRADIOL (E_2) IN PRE-TREATMENT AND 'CLOMID' TREATMENT CYCLES

SUBJECT	LENGTH OF LUTEAL PHASE (DAYS)		PROG.(ng)		17 OHP(ng)		E_2(pg)	
	PRE-TREATMENT	'CLOMID' TREATMENT	PRE-TREATMENT	'CLOMID' TREATMENT	PRE-TREATMENT	'CLOMID' TREATMENT	PRE-TREATMENT	'CLOMID' TREATMENT
Mrs. B.	16	16	68.3	120.5	22.6	26.1	1710	1380
Mrs. C.	15	15	49.2	137.8	27.1	32.6	1330	4900
Mrs. T.	16	14	71.8	65.4	17.9	12.1	1195	1760
Mrs. R. Cycle 1	14	14	55.4	30.7	20.9	14.5	2670	2268
Cycle 2		12		89.4		18.9		2305
Mrs. Mc.	14	14	78.2	89.9	14.6	13.5	2395	4205

TABLE 6. 2. COMPARISON OF LUTEAL PHASE PEAK HEIGHTS OF PROGESTERONE, (PROG.), 17α-HYDROXYPROGESTERONE (17 OHP), AND OESTRADIOL (E_2) IN PRE-TREATMENT AND 'CLOMID' TREATMENT CYCLES.

SUBJECT	LENGTH OF LUTEAL PHASE (DAYS)		PROG. (ng)		17 OHP (ng)		E_2 (pg)	
	PRE-TREATMENT	'CLOMID' TREATMENT	PRE-TREATMENT	'CLOMID' TREATMENT	PRE-TREATMENT	'CLOMID' TREATMENT	PRE-TREATMENT	'CLOMID' TREATMENT
Mrs. B.	16	16	6.9	14.4	2.6	3.3	155	165
Mrs. C.	15	15	5.9	20.0	2.7	3.7	160	595
Mrs. T.	16	14	9.3	16.1	2.1	1.9	200	280
Mrs. R.								
Cycle 1	14	14	6.5	4.0	2.3	1.3	245	280
Cycle 2		12		16.0		2.7		420
Mrs. Mc.	15	15	9.0	10.4	1.7	2.0	200	495

FIG. 6. VIII THE MEAN LEVELS OF LH ± S.D. (n = 6) IN INFERTILE WOMEN DURING A 'CLOMID' TREATMENT CYCLE COMPARED TO THE NORMAL RANGE.



normal range and are shown in Fig. 6. IX. The treatment range tended to be lower than the normal range in the early follicular and the late luteal phase. The treatment range was very wide at mid-cycle and the mean treatment level was higher than the normal mean level.

The mean oestradiol levels $\pm$ S.D. (n = 6) were compared to the normal range and are shown in Fig. 6. X. Follicular phase levels were similar in the two ranges. The mean mid-cycle peak height was greater in the treatment cycle than the normal cycle. In the luteal phase, the mean peak height was greater in the treatment cycle than in the normal cycle. Peak levels were reached by the same day in the two ranges. The treatment range was wider than the normal range in the luteal phase.

The mean 17α-hydroxyprogesterone levels $\pm$ S.D. (n = 6) were compared to the normal range and are shown in Fig. 6. XI. The ranges were similar throughout and did not differ markedly at any point.

The mean progesterone levels $\pm$ S.D. (n = 6) were compared to the normal range and are shown in Fig. 6. XII. The levels in the two ranges were similar in the follicular phase. The mean treatment level rose significantly by day 0, a rise which was not apparent in the normal mean cycle. The rate of

FIG. 6. IX THE MEAN LEVELS OF FSH ± S.D. (n = 6) IN INFERTILE WOMEN DURING A 'CLOMID' TREATMENT CYCLE COMPARED TO THE NORMAL RANGE.

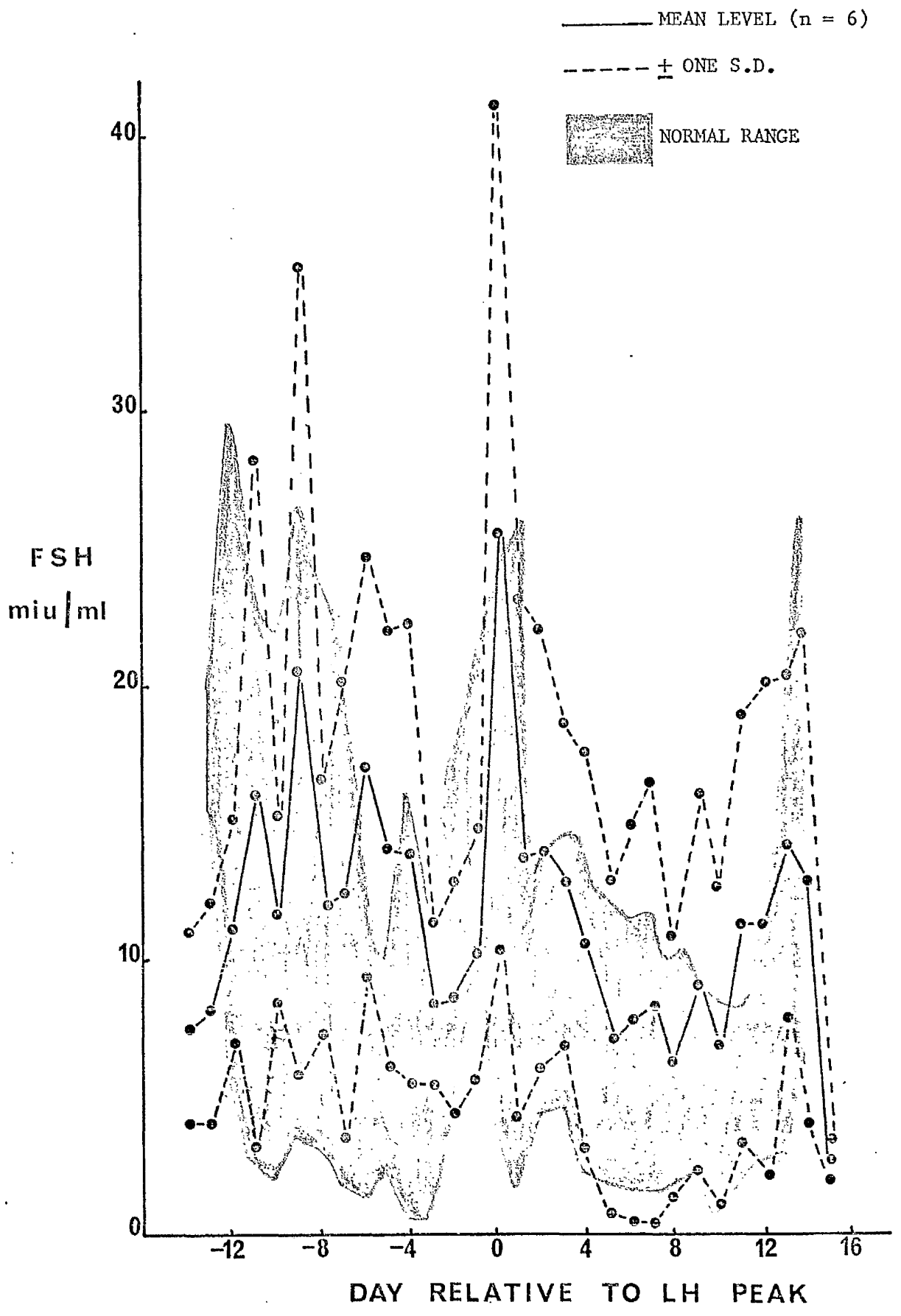


FIG. 6. X THE MEAN LEVELS OF OESTRADIOL (E_2) ± S.D. (n = 6) IN INFERTILE WOMEN DURING A 'CLOMID' TREATMENT CYCLE COMPARED TO THE NORMAL RANGE.

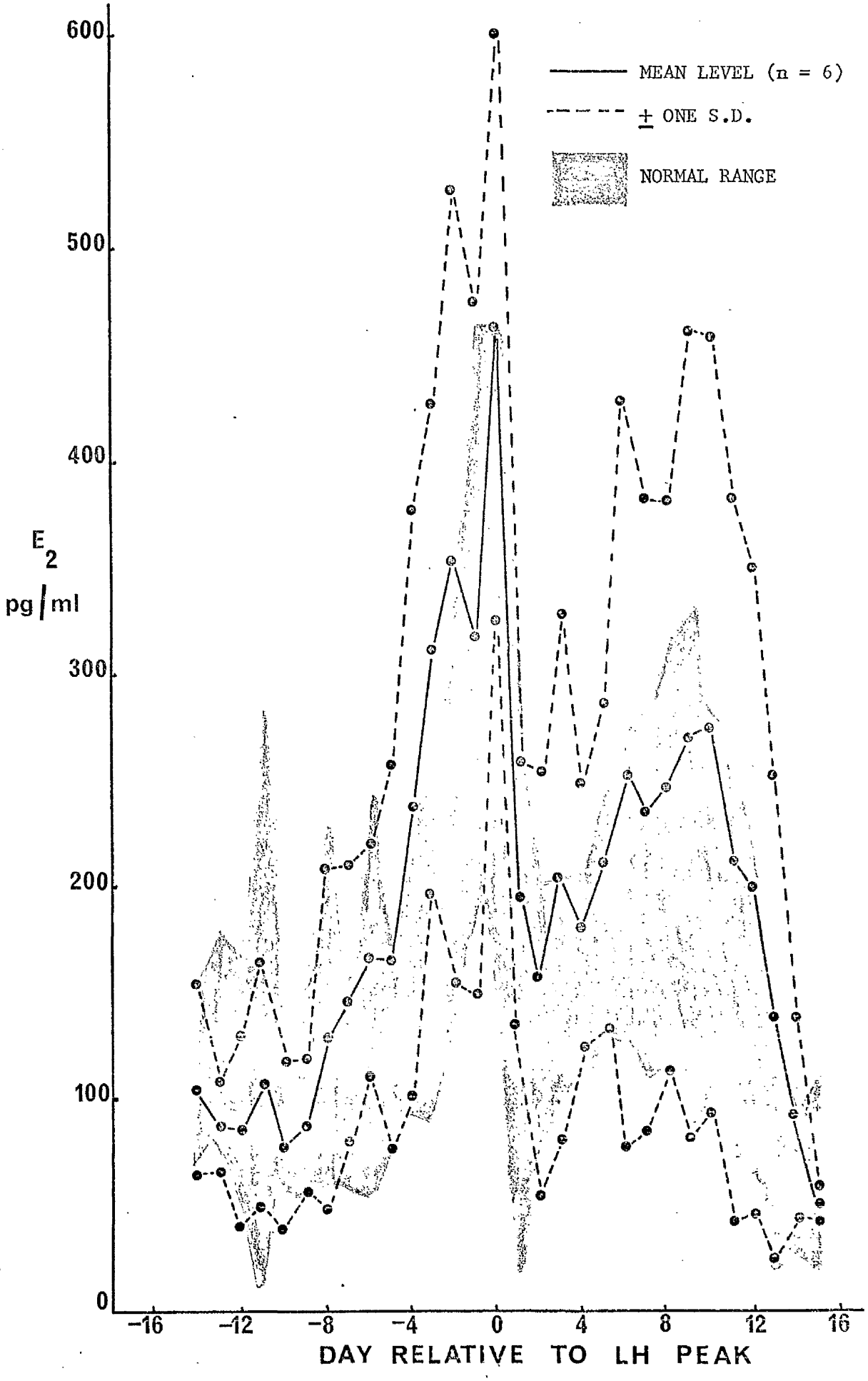


FIG. 6. XI THE MEAN LEVELS OF 17α-HYDROXYPROGESTERONE (17 OHP) ± S.D. (n = 6) IN INFERTILE WOMEN DURING A 'CLOMID' TREATMENT CYCLE COMPARED TO THE NORMAL RANGE.

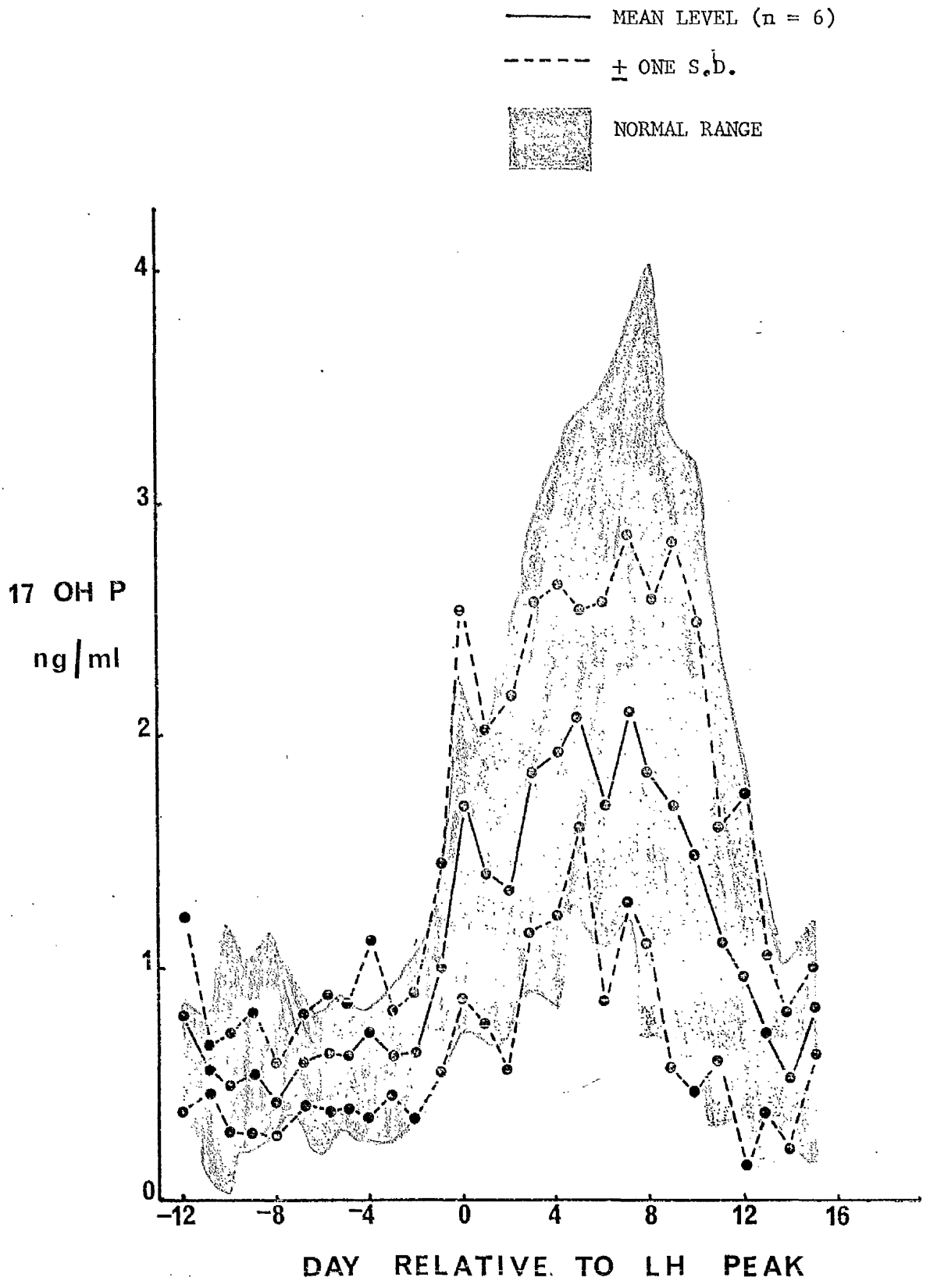
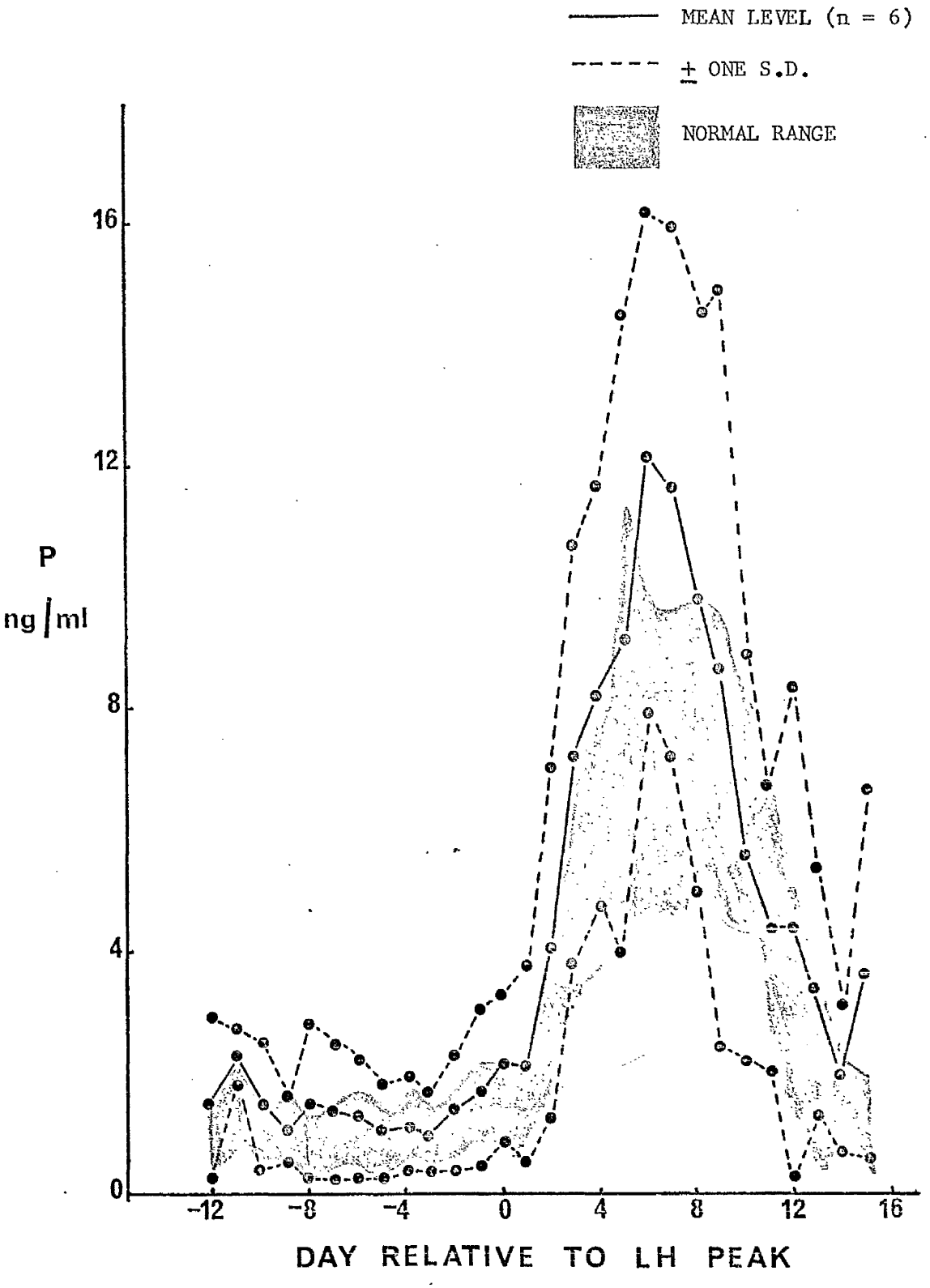


FIG. 6. XII THE MEAN LEVELS OF PROGESTERONE (P) ± S.D. (n = 6) IN INFERTILE WOMEN DURING A 'CLOMID' TREATMENT CYCLE COMPARED TO THE NORMAL RANGE.



progesterone increase in the luteal phase was similar in the two ranges, peak heights being attained by the same day. However, the mean peak levels in the treatment cycle were higher than in the normal cycle. Mean treatment levels were higher than mean normal levels on the final day of the cycles.

CHAPTER 7

DISCUSSION

DISCUSSION

PART I

CPB AND RIA METHODS

The reliability of a displacement analysis method is generally assessed in terms of four basic criteria: precision, accuracy, sensitivity and specificity. The most difficult of these four criteria to evaluate is perhaps specificity. This is also the most important criterion since a method with insufficient specificity will be of limited value even if it is satisfactory with regard to all other criteria. Sensitivity is an important consideration in the development of assays for hormones which are present in relatively small quantities in the fluid to be assayed. Precision and accuracy are important for reliability and reproducibility and must be attained through standardisation of conditions and constantly assessed when the method is in routine use.

The key to achieving specificity and sensitivity lies jointly in the choice of a binding protein and in the steps taken to purify the hormone prior to assay. The separation of free and protein bound hormone constitutes an important step in displacement analysis procedures and must be adequate

and reproducible in order to obtain acceptable sensitivity and precision. Methodological 'blank' has been traditionally the greatest single problem in development of such assays and must be insignificant if all four criteria are to be satisfactory.

THE BINDING PROTEIN

The most important consideration in the development of a displacement analysis is the production and isolation of a suitable binding protein. This protein should be capable of binding reversibly to one compound or to a group of chemically related compounds. There are three main sources of such proteins for the measurement of steroid hormones, the plasma, which contains specific proteins for hormones (Antoniades, 1961), extracts of tissue from steroid hormone target organs and antibodies raised to steroid-protein conjugates. The plasma proteins are easily obtained and have been used in the assay of several hormones, for example corticosteroids (Murphy et al., 1963), progesterone and 17α-hydroxyprogesterone (Strott and Lipsett, 1968), testosterone (Rosenfield et al., 1969). However, the poor specificity of the binding to such proteins has led to the development of methods using more specific proteins from steroid target organs. Uterine cytosol, for example,

has been used to measure oestrogens (Corker et al., 1970; Korenman et al., 1970). Such proteins however, are difficult to prepare without loss of binding activity and are often unstable on storage. Antibodies have been utilised in recent years for the assay of many steroid hormones since they combine specificity and sensitivity with stability on storage. Once a suitable antiserum has been produced, a single bleed may provide sufficient antiserum for many assays over several years. In the present work, two of these groups of proteins have been utilised; the plasma protein, CBG, to measure progesterone and 17α-hydroxyprogesterone and an antiserum riased to oestradiol-17β-6 (O-carboxymethyl)oxime-bovine serum albumin to assay oestradiol-17β. These two proteins have advantages for their chosen uses

CBG is theoretically capable of binding reversibly all 4-ene, 3-one steroids. However, different animal CBG's bind with different affinity to different steroids, and the choice of species must depend to some extent on the steroid being measured (Murphy, 1967). Westphal (1961) suggested a 'polarity rule' for binding of steroids to CBG. In theory the greater the polarity of the steroid, the greater is its binding affinity to CBG. In practice, however, this rule is not always obeyed. The association constant of the progesterone complex with human

CBG at 37°C is about three times as great as that of the similar cortisol complex, but progesterone interacts with rabbit CBG at 4°C with an affinity of approximately 40% of that shown by cortisol (Westphal, 1967). Guinea-pig CBG binds progesterone much more strongly than cortisol (Westphal, 1967).

Temperature also affects the binding between CBG and steroid. Evidence of greater dissociation at 37°C than at 4°C for the cortisol-human CBG complex was first reported by Slaunwhite and Sandberg (1959). Since then it has been shown that the association constants for corticosteroid complexes with human, rabbit and rat CBG are approximately twenty times greater at 4°C than at 37°C (Muldoon and Westphal, 1967; Chader and Westphal, 1968a; Chader and Westphal, 1968b). Murphy (1970) suggested that assays should be conducted between 4°C and 6°C because of the higher association constants at these temperatures and that temperatures should be controlled for reproducibility. In the present work, a temperature of 45°C has been used successfully to obtain dissociation of steroid from protein to allow the formation of a new complex, which then has a maximum association at 4°C (see p. 43).

The binding affinity between the corticosteroid hormones and CBG is also dependent upon the pH of the solution, a maximum

value being observed at approximately pH 8.0 (De Moor et al., 1963). The present work (Fig. 3. IV) confirms this observation. Daughaday and Mariz (1961) and Seal and Doe (1962) have reported irreversible denaturation of human CBG with loss of binding activity at pH 5.0 and below.

CBG's from several species have been used for assay of progesterone. Dog CBG has been used (Martin et al., 1970), but Reeves et al., (1970) compared the sensitivity of displacement in human and dog plasma for amounts of progesterone less than 1 ng and reported that only human CBG provided the required sensitivity for a precise and accurate assay at these low levels of the hormone. In the present study human CBG has been used to obtain a highly sensitive assay for progesterone and more especially 17α-hydroxyprogesterone which is present in plasma in lower concentration than progesterone in the luteal phase of the menstrual cycle.

The administration of oestrogen and pregnancy increase the concentration of CBG in peripheral plasma (De Moor et al., 1962). Late pregnancy plasma has been used to assay progesterone (Martin, 1970b) but was insensitive probably due to the large amounts of endogenous steroid present in the plasma. Plasma from women taking oral contraceptives has been used by Johansson (1970) but his standard protein solution did not keep well for

longer than 48 hours and ageing was variable. However, examination of the method reveals that he prepared the SPS in distilled water, and not in buffer. As distilled water, especially if previously deionized is slightly acidic (pH5-6) gradual denaturation of the protein may have occurred. It is now not possible to use plasma from women taking oral contraceptives for binding as the new low oestrogen 'pill' does not produce a suitable titre of CBG (Coutts, 1972). The work of Johansson (1970) was performed using the plasma from women taking high oestrogen 'pills'.

In the present work CBG was obtained in the plasma of a post-menopausal woman treated with ethinyl oestradiol and dexamethasone. Virtually the same process has been used by Reeves et al., (1970) who treated an ovariectomised woman with equine conjugated oestrogen. The plasma produced a sensitive SPS which was stable for up to three weeks when prepared in buffer at pH 8.0 and stored in a refrigerator. Such plasma can be obtained in large quantities by plasmaphoresis and stored at -15^{o}C for long periods of time (at least two years) without denaturation. It is not susceptible to denaturation by freezing and thawing at least three times.

Lipsett et al., (1970) and Reeves et al., (1970) have administered dexamethasone when preparing plasma containing

CBG to prevent the saturation of CBG with endogenous steroid. These authors found that this procedure increased the sensitivity of the CBG. Administration of dexamethasone following ethinyl oestradiol has been used in the present work despite Johansson's (1970) inability to demonstrate any improvement in the binding properties of his protein after its administration.

Displacement analysis methods rely theoretically on the ability of a non-radioactive compound to displace its radioactive isomer from the CBG. In practice, however, for the progesterone and 17α-hydroxyprogesterone assay, better initial binding and displacement was achieved by displacing $\left[^{3}H\right]$ corticosterone with progesterone or 17α-hydroxyprogesterone than when $\left[^{3}H\right]$progesterone or $\left[^{3}H\right]$ 17α-hydroxyprogesterone were used for initial binding. Approximately 20% greater binding and displacement was achieved by Martin (1970a). All CPB methods for progesterone have used $\left[^{3}H\right]$ corticosterone (e.g. Neill et al., 1967; Johansson, 1969; Reeves et al., 1970) with the exception of that of Yoshimi and Lipsett (1968) who used $\left[^{3}H\right]$ progesterone for initial binding. The better results obtained using $\left[^{3}H\right]$ corticosterone can be accounted for by the fact that different CBG's bind with different affinity to different steroids depending upon the temperature and the concentration of substances present. Westphal (1967)

has thus shown that for human CBG at $4^{o}C$ and at a low concentration, progesterone will be bound more strongly than corticosterone. This phenomenon has never been successfully explained despite the fact that this observation contradicts Westphal's (1961) 'polarity rule'.

Tritiated steroids are most commonly used in displacement analysis. $\left[^{14}C\right]$ compounds could not be considered for the present work since the specific activity of the steroids labelled with this isotope is too low to give the required sensitivity. Midgley and Niswender (1970) suggested that $\left[^{125}I\right]$ or $\left[^{131}I\right]$ labelling has great advantages over $\left[^{3}H\right]$ labelling for displacement analyses because of the higher specific activity and better counting efficiency. However, this is questionable since it is not possible to label steroids, apart from oestrogenic onces, directly with the $\left[I\right]$ atom, and the compounds produced are thus less stable than those labelled with $\left[^{3}H\right]$. More elaborate precautions are also necessary in the handling of $\left[I\right]$ labelled compounds than $\left[^{3}H\right]$ labelled onces and their shorter half-lives necessitate more frequent labelling. $\left[I\right]$ labelled steroids were not considered for the present work as $\left[^{3}H\right]$ labelling provided the required sensitivity.

The use of an antibody for measurement of oestradiol is particularly suitable as the levels of circulating oestradiol

in peripheral plasma during the menstrual cycle are very low. Radioimmunoassay can provide a highly sensitive and specific assay at sub-picomole per ml levels. Uterine cytosol has been used in CPB assay of oestradiol (Corker et al., 1970), but has the disadvantages that it is difficult to prepare and unstable on storing and binds not only other oestrogens but also other, not necessarily steroidal, oestrogenic compounds (Jensen et al., 1968). Radioimmunoassays have been used by many workers to measure oestradiol (Abraham et al., 1970; Mikhail et al., 1970; Exley et al., 1971; Wu and Lundy, 1971) although different workers have used different antisera.

To render a steroid molecule antigenic it must be coupled to a high molecular weight protein such as bovine or rabbit serum albumin. The greatest specificity of antibody formation is generally obtained by conjugating the steroid through a position in the steroid nucleus remote from the distinguishing functional groups of that steroid. Oestradiol has been conjugated to BSA through the '3' position (Thorneycroft et al., 1970), the '17' position (Wu and Lundy, 1971) and the '6' position of the steroid nucleus (Exley et al., 1971). An antiserum produced to a conjugate linked at the '6' position is most specific and has been employed in the present study.

However, Walker et al., (1973) have shown that specificity depends not only on the site of conjugation but also on the carrier protein and on the immunisation procedure.

The choice of antisera depends upon the estimations to be carried out. A relatively non-specific antiserum, such as one produced to a conjugate coupled at the '17' position, is useful if more than one oestrogen is to be measured in the same plasma sample. The oestrogens can then be separated by chromatography prior to assay of the different fractions with the same antiserum. The '6' linked conjugate produced an antiserum suitable for the present work where only oestradiol was to be measured. The high specificity rendered a chromatographic purification step unnecessary (Table 3. 4.)

In order to obtain an antiserum of high titre for measuring oestradiol, the antiserum can be treated with BSA (Campbell et al., 1964) to precipitate antibodies directed against the carrier protein. Treatment with Rivanol (Horesji and Smetana, 1956) increases the sensitivity of the antiserum by removing albumin from the serum, thus limiting the non-specific binding. Both of these treatments were used in preparation of the antiserum used in this study.

The attainment of equilibrium in displacement analyses is reported to take longer at higher dilutions and lower temperatures (Murphy, 1970) - seconds to minutes for assays using plasma proteins and hours to days for antibodies. The present work demonstrates that a shorter time (20 mins) is sufficient for equilibrium to be reached using this particular antibody (Fig. 3. XII). Abraham et al., (1970) report that the sensitivity and binding of oestradiol increases with time of incubation from 2 to 16-20 hours in a solid phase RIA system. The method reported here does not appear to show this phenomenon, although a careful study of sensitivity at different incubation times has not been undertaken. It is possible that this phenomenon varies with different systems and different antisera. A temperature of $4^{\circ}C$ was employed in the present work to obtain maximum association between steroid and protein.

The antiserum used was stable on storage, having been stored as a 1 in 5 dilution (following Rivanol treatment) for two years at $-15^{\circ}C$ without noticable deterioration of its binding properties. These properties were not affected by freezing and thawing of the antiserum up to two times. A solution of antiserum could be stored for up to two weeks at $4^{\circ}C$, without deterioration, provided the solution contained thimerosal.

The addition of thimerosal was found necessary in the preparation of all solutions used for the RIA, presumably as it inhibited the growth of bacteria in the solutions which contain gelatine. Any other antibacterial agent would presumably also have been effective in this way.

EXTRACTION AND PURIFICATION OF STEROIDS

Purification of the steroid prior to assay is also important in obtaining specificity and sensitivity. Extraction of the steroid with a suitable solvent is the initial step in the purification. Progesterone has been extracted using a number of solvents including light petroleum (Johansson, 1969; Reeves et al., 1970) and ethyl acetate (Martin et al., 1970). Light petroleum extracts up to 90% of progesterone (Murphy, 1967) and only relatively small amounts of other steroids (Lipsett et al., 1970). This solvent is therefore unsuitable for the present work where 17α-hydroxyprogesterone must also be extracted. Ethyl acetate has been used by Martin et al., (1970) for extracting progesterone. However diethyl ether was found to be a more suitable extracting solvent in the present work because of the efficiency of extraction of both progesterone and 17α-hydroxyprogesterone and the ease of evaporation. The latter consideration is of importance practically, both when high demands are made on limited evaporation equipment in the

laboratory and with regard to total assay time. Lipsett et al., (1970) confirm that diethyl ether gives a high yield extraction for steroids of polarity equal to or less than 17α-hydroxy-pregnenolone. Several workers have used more than one extraction (Lipsett et al., 1970; Reeves et al., 1970) but in the present study a single extraction gave good quantitative removal of progesterone and 17α-hydroxyprogesterone from the plasma.

Diethylether has frequently been used for extraction of oestrogens (Korenman et al., 1970; Mikhail et al., 1970; Abraham et al.,1970; Wu and Lundy, 1971). Several workers have made the plasma alkaline either with carbonate buffer, pH 9.5 (Mikhail et al., 1970) or with NH_4OH (Korenman et al., 1970) before extraction to increase extraction efficiency. However ether extraction at neutral pH, gave quantitive recovery in the present study. Purification of the steroid involves separation from two types of substance: other steroids with high affinity for the binding protein and unknown, probably non-steroidal materials, that either compete for, or inactivate steroid binding sites on the protein. Such materials are present in many solvents and chromatographic materials. Chromatographic separation is commonly used to separate the steroid

of interest from other interfering steroids. Thin layer chromatography (Strott and Lipsett, 1968), Sephadex columns (Murphy, 1970), paper chromatography (Reeves et al., 1970; Martin et al., 1970) and celite columns (Stone et al., 1971) have been used for the purification of progesterone and 17α-hydroxyprogesterone. TLC provides a suitable purification of progesterone and partial purification of 17α-hydroxyprogesterone but many workers report 'blank' problems even after careful and prolonged washing, and poor recoveries from the TLC plates (Murphy, 1970; Reeves et al., 1970). Both Sephadex columns (Murphy, 1970) and Celite columns (Stone et al., 1971) have been reported to give this purification without a 'blank' problem. However, paper chromatography was the method of choice in the present study for several reasons. Firstly, the paper could be thoroughly washed in methanol before use thus eliminating any blank. Secondly, the method was reasonably quick; 36 samples could conveniently be applied to paper in two hours and the papers could be developed overnight using an automatic solvent changer and time clock device. Thirdly, paper chromatography systems in general give very reproducible R_F values for steroids. Fourthly, elution from the paper was quantitatively efficient and simple using the syringe and bent needle technique (p. 23).

The separation of 17α-hydroxyprogesterone from testosterone poses a greater problem. A suitable solvent system, for either paper or TLC has not been devised at present for complete separation of these two steroids. Strott and Lipsett (1968) used a 24 hour acetylation technique to acetylate the testosterone. This is a lengthy procedure. A five hour acetylation has been found to give satisfactory results in the present study (Table 3. 2). Holmdahl and Sjövall (1971) separated 17α-hydroxyprogesterone from testosterone using liquid gel chromatography on a hydrophobic hydroxy-alkoxy propyl Sephadex derivative. However, whilst separation was achieved between 17α- hydroxyprogesterone and testosterone, 17α-hydroxyprogesterone was not separated from androstenedione which may interfere in the CPB assay.

Further investigation of the 'cross reaction' of testosterone in the CPB assay for 17α-hydroxyprogesterone has shown that the acetylation may be unnecessary for measurement in normal female plasma. As shown in Fig. 3. V. testosterone appears to displace $\left[^{3}H\right]$ corticosterone from CBG but this experiment was carried out using two times excess of testosterone over 17α-hydroxyprogesterone. Such conditions may be found in the follicular phase of the menstrual cycle. Judd and Yen (1973) have published values for testosterone in the follicular

phase of the menstrual cycle of approximately 0.4 ng/ml whilst levels of 17α-hydroxyprogesterone as low as 0.2 ng/ml have been observed in the present study. CBG binds testosterone to a much lesser extent than corticosterone or 17α-hydroxyprogesterone (Lebeau et al., 1969). Competition for binding sites on CBG may therefore be negligible in the presence of both corticosterone and 17α-hydroxyprogesterone especially when the latter is in excess over testosterone as in the luteal phase of the menstrual cycle. A preliminary experiment has been performed in which several samples from a pool of menstrual cycle plasma have been assayed for 17α-hydroxyprogesterone with and without acetylation. No significant difference was found in the two sets of results. Measurement of 17α-hydroxyprogesterone may therefore be possible without acetylation but careful consideration must be given to the levels of the two hormones expected in the plasma being assayed. The levels of testosterone in several types of plasma (e.g. luteal phase menstrual cycle), may be so low in relation to levels of 17α-hydroxyprogesterone as to be negligible and unlikely to interfere.

The extraction and purification procedures employed gave good overall recovery for progesterone. The lower recovery of 17α-hydroxyprogesterone was possibly due to losses incurred through the acetylation and second chromatography. However,

the lower recovery of 17α-hydroxyprogesterone may reflect less efficient elution of the more polar 17α-hydroxyprogesterone from the paper. When 17α-hydroxyprogesterone was assayed without the acetylation and second chromatography steps a recovery of 70.5 $\pm$ 5.5% (n = 50) of added $\left[^3H\right]$ 17α-hydroxyprogesterone was obtained. This figure is somewhat lower than that for the progesterone assay (p. 37).

Complicated purification of the oestradiol extract was not required because of the high specificity of the antiserum. The only compound showing significant cross reaction was 6-oxo oestradiol but this has not beenreported in plasma in the non-pregnant woman. A qualitative assessment of the cross reaction ofoestrone and oestriol with the antiserum was made in Fig.3. IX. Whilst oestrone and oestriol appeared to displace $\left[^3H\right]$ oestradiol from the antiserum when present alone, Fig. 3. X showed that no such displacement occurred in the presence of oestradiol. The quantitative assessment of cross reaction (Table 3. 5) therefore probably has little bearing on the practical situation. Oestrone and oestriol appear to offer no competition for binding sites when present along with oestradiol, as in plasma. Absence of significant cross reaction has only been verified for equal concentrations of the three oestrogens.

However, the concentrations of unconjugated oestrone and oestriol are lower than the concentration of oestradiol both in the menstrual cycle and throughout pregnancy (Tulchinsky et al., 1972). The method is therefore probably suitable for measurement of oestradiol in pregnancy plasma also. Recently the presence of an unknown substance, in the plasma of women receiving treatment with 'Pergonal', which cross reacts with the antiserum has been suggested (Exley, 1973). If this substance is produced as a result of stimulation of the ovary it may also be present in the plasma of 'Clomid' treated women. However, it may alternatively be a substance produced due to the administration of high doses of 'Pergonal'. Exley (1973) has suggested that this compound may be 6-oxo oestradiol and work is at present in progress in this laboratory to investigate this suggestion. This observation draws attention to the necessity for checking carefully various types of plasma before setting up a routine assay.

METHODOLOGICAL INTERFERING FACTORS

One of the most notorious problems in displacement analyses is the removal of unknown, non-steroidal materials which compete with the steroid for binding sites on the protein. The problems of choosing suitable blank samples have been outlined (p. 45).

These problems have not been solved in the present work, as no suitable blank plasma has been obtained. 'Water blanks' have been used to estimate methodological interfering factors due to solvents and other materials used in the assay.

Murphy (1970) reported that most organic solvents give rise to positive blank values. These values vary with batches of solvent, are very unpredictable, can be removed by distillation but may reappear on storage. Diethyl ether used for RIA showed this blank phenomena and was redistilled frequently to maintain low blanks. A blank is only subtractable from determinations if the contamination is bound to the protein with the same affinity as the steroid, and since this is unlikely, blanks are probably not subtractable. To reduce blanks Murphy (1970) suggested plastic containers should not be used in contact with organic solvents and that glass-ware should be washed in an ultrasonic bath. Methodological interfering factors were reduced to a negligible level in both the CPB and RIA assays. This was achieved by using Murphy's (1970) suggestions. Solvents were redistilled twice before use and chromatography paper and glassware were meticulously washed. TLC which is notorious for producing high blank values (Crepy et al., 1969; Thompson et al., 1969; Murphy, 1970) was avoided.

The blank values in both CPB assay and RIA have decreased significantly since the assays were first developed, presumably due to a greater familiarity with the methods. The blank values quoted for the progesterone and 17α-hydroxyprogesterone assay were perhaps erroneously high. Blanks were sometimes found to have negative values - i.e. the percentage of $\left[^{3}H\right]$ corticosterone bound in these samples was greater than that of the zero point (0 ng of progestogen) of the standard curve. Such values could not be estimated and therefore were designated as zero in calculating mean blank values. The blank values obtained probably reflect scatter around zero. The blank in the oestradiol assay has never been reduced to zero despite meticulous care with solvents, glassware and general handling. However, the blank is consistent within an assay.

SEPARATION OF FREE AND BOUND STEROID

Successful and reproducible separation of free and protein bound steroid is a critical and often difficult aspect in such methods. Methods which have achieved success in displacement analyses are protein precipitation and ligand adsorption. Mayes and Nugent (1968) used ammonium sulphate to precipitate protein-steroid complexes whilst adsorption of free steroid has been

achieved using Florisil (Frick and Kincl, 1969; Liberti et al., 1970; Martin et al., 1970), Fuller's earth (Murphy, 1967), Sephadex (Kato and Horton, 1968) and dextran coated charcoal (Rosenfield et al., 1969).

Florisil has been used extensively but has serious drawbacks. Strott and Lipsett (1968) have noted differences between batches of Florisil and suggested that it may be necessary to try several. Murphy (1969) noted that some batches of Florisil were 'sticky' due to an electrical charge and that they contained excessive 'fines'. De Souza et al., (1970) experienced difficulty with two batches of Florisil; one batch began satisfactorily but deteriorated over a period of several months whilst the other, despite efforts to improve its performance, remained unusable. Martin (1970a) was also unable to obtain suitable batches of Florisil after publication of his work (Martin et al., 1970).

The separation to be achieved by this procedure is between steroid bound to protein with high binding affinity and steroid either in solution or bound to protein of high capacity and low binding affinity. The use of Sephadex to separate bound and free steroid is often unsatisfactory since low affinity proteins will often release steroid during passage through a Sephadex column (Lipsett et al., 1970). Yoshimi and Lipsett (1968) used Sephadex in a CPB assay for progesterone but discovered that Sephadex competed with CBG for progesterone (Lipsett et al., 1970).

Charcoal has long been established as an adsorbent material for separation of free and bound compounds in displacement analyses. Charcoal was originally proposed by Miller (1957) for a Vitamin B_{12} assay and later used by Herbert et al., (1964). Rosenfield et al., (1969) demonstrated that charcoal could be combined with dextran to give a more viscous, stable phase which did not mix until shaken. Ekins (1968) and Hunter and Ganguli (1971) have emphasised that satisfactory separation of free and bound compound could be achieved using charcoal alone. However, where adsorption of free hormone is required with little adsorption of complex, it may be preferable to use dextran coating since the dextran may act as a 'molecular sieve' (Gottlieb et al., 1965), excluding the complex while permitting adsorption of free hormone. Binoux and Odell (1973) have criticised this theory and shown that, using thyroid stimulating hormone RIA as a model, dextran coating does not provide any 'molecular sieving' and the best conditions for separation of free and bound hormone occur when an appropriate concentration of uncoated charcoal is present. These authors have shown the same to be true in an assay for dihydrotestosterone.

Although no detailed study of conditions was undertaken,

dextran coated charcoal was used satisfactorily in both the CPB assay and RIA. This step could perhaps have been improved by a more careful study of the conditions but this was not merited by practical considerations.

De la Pena and Goldzieher (1971) have compared the accuracy and sensitivity of Sephadex, dextran coated charcoal and ammonium sulphate as agents for separating free and bound steroid in a CPB assay for testosterone. They concluded that although accuracy and sensitivity of the three methods were identical, the reproducibility and simplicity of the ammonium sulphate method were superior and commended it as the method of choice. However, the method of choice in an individual laboratory will be dependent upon availability of equipment and personal preference. The technique chosen must be performed under carefully controlled conditions of temperature and time in order to obtain reliable and precise results. The addition of gelatine solution prior to charcoal has been used by several workers (e.g. Wu and Lundy, 1971). Its mode of action is obscure. It is concievably useful in assays employing protein precipitation as the method of free and bound hormone separation since addition of extra protein may increase the precipitation of the very small amounts of protein present in the assay system. However many workers, without explanation,

employ gelatine addition where precipitation of free steroid is carried out. It has been used in the present work and has been found useful in apparently preventing 'stripping' of the bound steroid by the charcoal. It could conceivably provide a 'molecular sieving' mechanism and prevent adsorption of bound steroid by the charcoal even though this is dextran coated. Herbert et al., (1964) provided evidence in support of this hypothesis. They found that, in an assay for Vitamin B_{12}, charcoal saturated with protein would take up only free vitamin but that charcoal alone would also take up vitamin B_{12} bound to its binding protein.

GENERAL CONSIDERATIONS AND CONCLUSIONS

The methods developed for assay of progesterone, 17α-hydroxyprogesterone and oestradiol are satisfactory for the purpose for which they were developed and include certain features which make them more efficient and quicker to perform than other published methods, without loss of specificity or sensitivity. Little elaborate equipment is required and the methods, once established, require little routine maintenance, except due care in general cleanliness, redistillation of solvents, washing of chromatography papers and glassware.

There is no doubt that the 'rapid' CPB method of Johansson (1969) is quicker for the measurement of progesterone but specificity is sacrificed. The light petroleum used by Johansson extracts 90% of progesterone (Murphy, 1967) and only small amounts of other steroids. However, these other steroids interfere in the CPB assay. Light petroleum only extracts 13% of 17α-hydroxy-progesterone (Johansson, 1970), and could therefore not be used for a method combining assay of the two hormones. Johansson's (1969) method is useful for routine, clinical determinations but unsuitable when specificity and accuracy are essential as in research work. Johansson's method is inaccurate in the follicular phase of the menstrual cycle but during the luteal phase gives good agreement with other methods. It also relies on obtaining suitable light petroleum to achieve the selective extraction. There are reports that only certain batches will behave in this way (Murphy, 1967; Johansson, 1970).

The working ranges of the standard curves for progesterone and 17α-hydroxyprogesterone employed in the present study enable both hormones to be measured in 1 ml of plasma throughout the entire menstrual cycle. This is an important consideration as the use of different volumes at different stages of the cycle or different volumes for the two hormones introduces greater vari-

ability into the assay. The importance of the working range is demonstrated by De Souza et al., (1970) whose standard curve for progesterone only ranged from 0 to 1 ng. Consequently several dilutions of each sample were assayed to ensure that the unknown sample fell in the correct range for measurement.

Strott and Lipsett (1968) have reported a CPB assay for the combined measurement of progesterone and 17α-hydroxyprogesterone. However, their method has several drawbacks which have been eliminated in the method developed here. Firstly their method requires 3.5 ml of plasma due to its lower sensitivity. Consequently 25 ml of ether is required for extraction of steroids from this large volume of plasma. Evaporation of large volumes of solvent is a time consuming process. They also employ TLC for purification of steroids. This is now known to introduce methodological interfering factors in the assay. They also employ a 24 hour acetylation step, which has been reduced to 5 hours in the present work.

The working range for the oestradiol assay enables this hormone to be measured throughout the entire menstrual cycle in 0.2 ml of plasma. It is important that the volumes of plasma used are as low as is practical, since it is often difficult to obtain, in a single sample, sufficient plasma for all of the

assays to be carried out. Thus the combination of assay of progesterone and 17α-hydroxyprogesterone is particularly useful.

RIA methods for the measurement of progesterone (Youssefnejadian et al., 1972a) and 17α-hydroxyprogesterone (Youssefnejadian et al., 1972b) have been described. By using a RIA, the volume of plasma required could be reduced. However, it is questionable whether such a sensitive method is suitable for assay of progesterone and 17α-hydroxyprogesterone in human menstrual cycle plasma. Firstly, the levels of 17α-hydroxyprogesterone and more especially progesterone are such that either very small initial volumes of plasma or fractions of an extract of a larger volume would be required for the assay. Either of these procedures may increase the errors involved, by multiplication. Secondly, a combined RIA for progesterone and 17α-hydroxyprogesterone would be complicated by the fact that higher concentrations of progesterone than 17α-hydroxyprogesterone are present in the luteal phase of the cycle. This would involve removing different volumes of extracts for assay of the two hormones. These problems would be magnified when dealing with pregnancy plasma when the concentration of progesterone vastly exceeds that of 17α-hydroxyprogesterone. The time taken for the assay would not be reduced considerably with RIA since the two hormones would require

chromatographic separation to measure both in the same aliquot of plasma. Antisera are available, however, for 17α-hydroxy-progesterone, which do not cross react with testosterone (e.g. Youssefnejadian, 1972b) and the acetylation step could therefore be eliminated.

CPB is probably still the method of choice for the combined estimation of progesterone and 17α-hydroxyprogesterone in the human menstrual cycle and/or pregnancy plasma. 36 samples can be handled in a single assay in both the CPB assay and in the RIA for oestradiol. Thus samples from an entire cycle can be estimated, in the same assay, together with requisite 'blanks' and quality control samples. Samples have not been assayed in duplicate as this would be impractical in a single assay. However, assays have been repeated when blank and/or quality control results failed to reach the desired standard.

Many methods (both CPB and RIA) have been published for the estimation of these steroids. The methods differ mainly in the protein used for binding, the purification of the steroid and the separation of free and bound steroid. Different workers encounter different problems with various techniques so that undoubtedly the method of choice in a specific laboratory will differ from that in another. It is likely that any laboratory will make modifications to existing methods and it is of utmost

importance that the method used is evaluated thoroughly, carefully controlled, and normal ranges established in each particular laboratory.

PART II

A. THE NORMAL MENSTRUAL CYCLE

i) The General Outline of Events

The present study demonstrates the patterns of ovarian and gonadotrophin hormones throughout the normal menstrual cycle. The mean hormone patterns, shown in Figs. 4. II to 4. VI, give a good general outline of ovarian pituitary inter-relationships. The mean patterns however may mask some of the more subtle inter-relationships, seen in individual cycles, a knowledge of which may be essential in understanding the regulation of the menstrual cycle.

Presentation of the mean cycles in this study is necessary firstly to obtain normal ranges for each hormone throughout the cycle and secondly, for comparison with patterns in infertile subjects. The mean patterns and levels of hormones, shown in Figs. 4. II to 4. VI. are in good agreement with those reported by other workers (e.g. Vande Wiele et al., 1970; Mishell et al., 1971; Abraham et al., 1972). The mean levels $\pm$ one S.D. demonstrate clearly the large individual variation seen in all hormones throughout the cycle.

The only major peak of LH, which occurred in all normal cycles studied was at mid-cycle (day 0). The peak level varied in magnitude from 15 to 32 m.i.u./ml. FSH levels were generally higher in the follicular phase than in the luteal phase. A mid-cycle peak occurred in all normal cycles. Levels began to rise towards the end of the cycle.

Oestradiol levels fluctuated in the follicular phase and rose steadily from about day -4 reaching a peak on day -1. A drop occurred after day 0, followed by a second rise of less magnitude than the mid-cycle rise in the luteal phase. Levels fell to early follicular phase values by the end of the cycle.

Progesterone levels were low in the follicular phase and rose after day 0 to a peak in the luteal phase. Levels fell to follicular phase values by the end of the cycle.

17∝-hydroxyprogesterone levels were low in the follicular phase and rose to a peak on day 0, coincident with the LH peak. They dropped slightly after this peak, and rose to a second peak of greater magnitude than the mid-cycle peak in the luteal phase. Levels fell to follicular phase values at the end of the cycle.

These results support the general hypothesis proposed by Vande Wiele et al., (1970) explaining the regulation of the menstrual cycle. High levels of FSH at the beginning of the

cycle stimulate follicular growth and maturation. This is reflected in increased output of oestradiol which, after reaching a certain level probably stimulates the release of LH, which induces ovulation. The formation of a corpus luteum is reflected by increased output of progesterone, 17α-hydroxyprogesterone and oestradiol. However, this appears to be an over-simplification of the situation. The hypothesis does not offer explanations of several phenomena such as the role or source of the mid-cycle peak of 17α-hydroxyprogesterone, the role of FSH at mid-cycle or of the mechanism of gonadotrophin release. In the present study, the sequence of events in the individual cycles has been examined to attempt to find answers to these problems.

ii) The occurrence of a Mid-cycle rise in 17α-hydroxyprogesterone

Strott et al., (1969) have reported the pattern of 17α-hydroxyprogesterone in the menstrual cycle. These authors reported a mid-cycle rise in all cycles studied. Some cycles displayed a discrete mid-cycle peak. The present study supports these observations, although the magnitude of this mid-cycle peak, or rise, is not as great as that reported by Strott et al., (1969). Discrete peaks were only observed in 5 out of 8 normal

cycles in the present study. Holmdahl and Johansson (1972) also reported that a discrete mid-cycle peak was not apparent in every cycle, but a significant increase in 17α-hydroxyprogesterone was always noted by the day of the LH peak. Holmdahl and Johansson (1972) also reported that the peak occurred on the same day as the mid-cycle oestradiol peak in the mean of 10 cycles. The present study shows the mid-cycle peak to occur on the day after the oestradiol peak in the mean of 8 cycles. This is in agreement with the findings of Abraham et al., (1972). However, individual cycles exhibit much variation with regard to this peak. In some, the peak occurs on the day before the LH peak and in others on the day of the LH peak.

These findings demonstrate that in a normal menstrual cycle, a discrete mid-cycle peak of 17α-hydroxyprogesterone is not always apparent. However, a rise in 17α-hydroxyprogesterone is always noted by the time of the LH peak but not before LH begins to rise. This pattern of 17α-hydroxyprogesterone suggests either that this steroid has some role in ovarian function or that its rise is merely reflecting some physiological process, possibly follicular maturation, as the rise corresponds to that of oestradiol. An investigation of the source of this mid-cycle 17α-hydroxyprogesterone may help to elucidate this problem.

iii. The Source of 17α-hydroxyprogesterone at mid-cycle

The similarity in patterns between 17α-hydroxyprogesterone and oestradiol at mid-cycle led Strott et al., (1969) to postulate that 17α-hydroxyprogesterone was produced by the theca interna cells of the developing follicle and secreted as an intermediate on the pathway to oestradiol. Ryan et al., (1968) showed that only theca cells could produce oestradiol in vitro from acetate and that these cells could also produce 17α-hydroxyprogesterone. These findings were consistent with Strott et al.'s., (1969) hypothesis. However, several observations suggest that the secretion of oestradiol and 17α-hydroxyprogesterone in the follicular phase and at mid-cycle may be unrelated. Firstly, the levels of oestradiol fluctuate greatly whilst levels of 17α-hydroxy-progesterone are fairly constant in the follicular phase. Fluctuations in the secretion of a hormone by the theca cells might be expected to be rapidly reflected in fluctuating peripheral levels of the hormone because of the rich blood supply to the theca cells. Follicular phase peaks of oestradiol are rarely associated with peaks of 17α-hydroxyprogesterone. Corresponding patterns throughout the follicular phase might be expected if the two hormones were related by a common pathway. Secondly, the levels of 17α-hydroxyprogesterone and oestradiol

at mid-cycle are not suggestive of such a relationship. The present study shows that the levels of 17α-hydroxyprogesterone are of the order of 10 times those of oestradiol. The hypothesis of Strott et al., (1969) would imply the leakage of 90% of the products of the pathway as an intermediate. Such a biosynthetic pathway would be inefficient and unlikely to function, unless the intermediate had some physiological role. The peak blood production rate of 17α-hydroxyprogesterone has been estimated at 4 mg/24 h and that of oestrogens as 0.3 mg/ 24 h (Strott et al., 1969). If 17α-hydroxyprogesterone was produced as an intermediate on the pathway to oestradiol, this would imply a rate limiting step at this point in oestradiol biosynthesis. Thirdly, 17α-hydroxyprogesterone and oestradiol do not always follow the same pattern after mid-cycle. Oestradiol levels always drop markedly whilst 17α-hydroxyprogesterone levels often continue to rise into the luteal phase.

Although most workers have sought to compare and relate oestradiol to 17α-hydroxyprogesterone it may be pertinent to consider also the pattern of progesterone secretion in the cycle. Although the mean cycle does not show a rise in progesterone levels before the LH peak, this is observed in several individual normal cycles. Three cycles exemplify this phenomenon. First,

in the cycle from subject KK (Fig. 4. IX) a significant increase in progesterone occurs from day -2 to 0; levels rise from 1.2 ng/ml to 2.4 ng/ml. At the same time 17α-hydroxyprogesterone levels rise from 1 ng/ml to 3 ng/ml. Secondly, in cycle 2 from subject PH (Fig. 4. XIII), progesterone levels rise from 1.2 ng/ml on day -3 to 2.0 ng/ml on day 0 and at the same time 17α-hydroxyprogesterone levels rise from 0.35 ng/ml to 1.6 ng/ml. Thirdly, in cycle 3 from subject PH (Fig. 4. XIV) progesterone levels rise from 1.0 ng/ml on day -2 to 2.0 ng/ml on day 0. 17α-hydroxyprogesterone levels rise from 0.1 ng/ml to 0.9 ng/ml over the same period. Progesterone levels do not rise significantly before LH begins to rise and progesterone is therefore unlikely to be involved in LH release. The levels of progesterone and 17α-hydroxyprogesterone and their concomitant rises are in much closer agreement than levels of oestradiol and 17α-hydroxyprogesterone at this time.

Other evidence suggests a pre-ovulatory rise in progesterone. Animal studies have shown that there is an increase in ovarian progesterone production before ovulation (Johansson et al., 1968). Niell et al., (1967) and Yoshimi and Lipsett (1968) suggested that no such increase occurred in women. However, Johansson and Wide (1969) studied in greater detail progesterone and LH

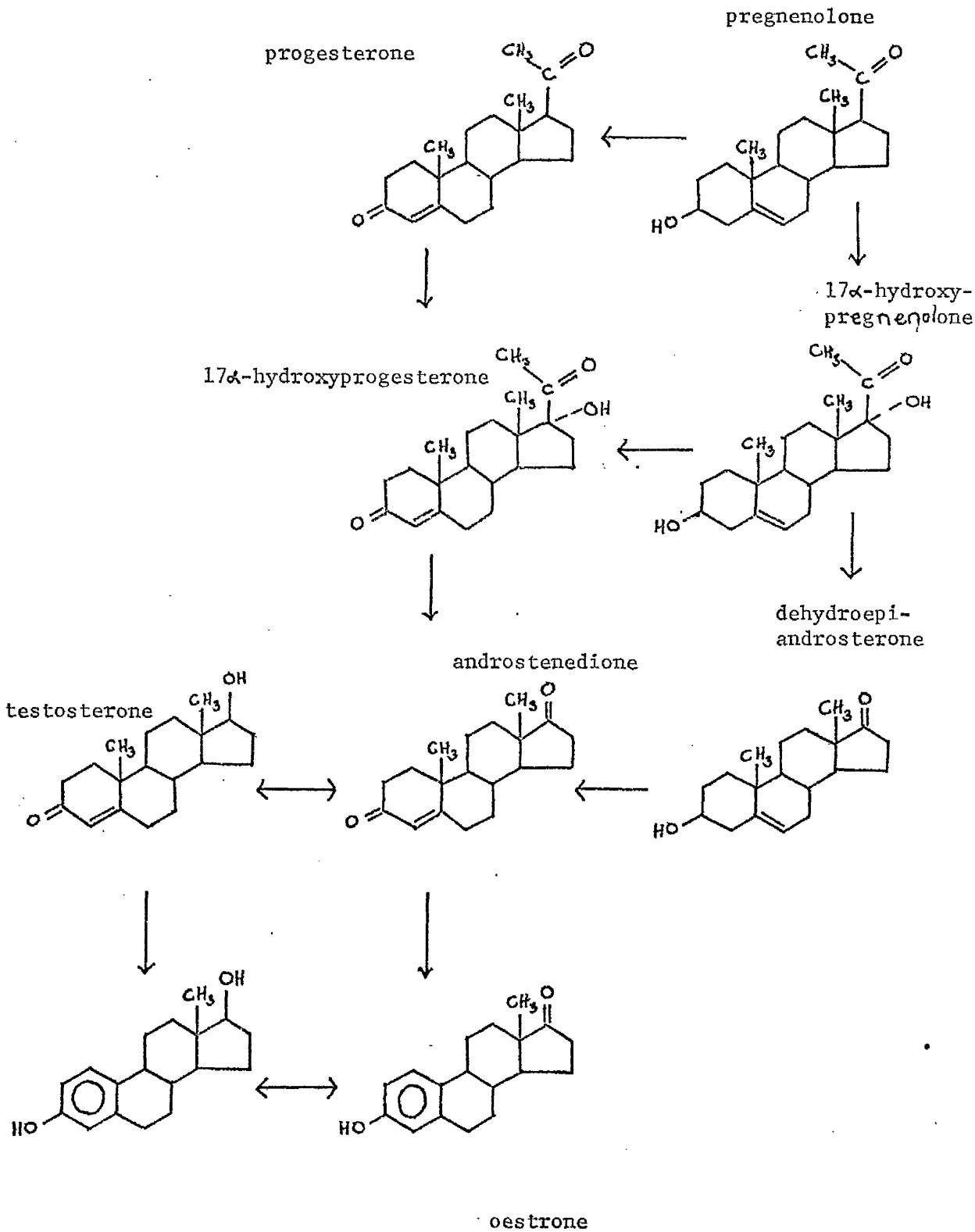
concentrations in plasma around the time of ovulation and have shown that a pre-ovulatory rise in progesterone occurs in some cycles but that a rise in progesterone was never observed before the first significant rise in LH. Further evidence in support of these findings has been given by Dhont et al., (1974). The results of the present study suggest a possible relationship between this pre-ovulatory rise in progesterone and that of 17α-hydroxyprogesterone. The progesterone rise is most likely due either to secretion by the ripe follicle before ovulation or to pre-ovulatory luteinization of ripe follicles. Mikhail reported a high concentration of progesterone (1967) and 17α-hydroxyprogesterone (1970) in ovarian vein blood draining from an ovary containing a ripe follicle.

It is possible that the increase in 17α-hydroxyprogesterone at mid-cycle could be derived by hydroxylation of progesterone produced either by the granulosa cells or the theca cells as they mature or become luteinized by the first rekase of LH. The increased production of both progesterone and 17α-hydroxy-progesterone often continuesinto the luteal phase. The diverging patterns of oestradiol and 17α-hydroxyprogesterone after mid-cycle, the former always dropping and the latter often rising and paralleling the increase in progesterone production, suggest

that oestradiol and 17α-hydroxyprogesterone are produced by different cell types. Hence it is suggested that the granulosa cells of the maturing follicle, possibly due to pre-ovulatory luteinization, are mainly responsible for the mid-cycle 17α-hydroxyprogesterone production, which is continued into the luteal phase, whilst oestradiol is produced by the theca cells which are not so readily luteinized. In vitro studies demonstrated that granulosa cells were more active in producing progesterone and 17α-hydroxyprogesterone from pregnenolone (Ryan and Petro, 1966) and from acetate (Ryan et al., 1968) than theca cells and are thus consistent with the above hypothesis. Hence it is suggested that granulosa cells produce 17α-hydroxyprogesterone via progesterone, and in amounts similar to that of progesterone at mid-cycle. The theca cells on the other hand metabolise pregnenolone to oestradiol via the alternate pathway involving 5-ene compounds. The amount of 17α-hydroxyprogesterone produced as an intermediate in this pathway might be expected to be small. These pathways are outlined in Fig. 7. I. The drop in oestradiol secretion after mid-cycle probably reflects a physiological change over from follicular to luteal production.

The fact that the pre-ovulatory rise in progesterone is not always apparent whilst the 17α-hydroxyprogesterone rise is always apparent may perhaps be due to individual variations in

FIG. 7. I PATHWAYS OF OVARIAN STEROID HORMONE METABOLISM. THE DIAGRAM DEMONSTRATES THE MAJOR PATHWAY VIA 4-ene, 3-one COMPOUNDS AND THE ALTERNATIVE PATHWAY VIA 5-ene, 3-ol COMPOUNDS.



the ability of the cells to 17α-hydroxylate progesterone. Alternatively this could be due to the accummulation of the precursor progesterone in granulosa cells which do not have a rich vascular supply. Such accummulation might lead to increased 17α-hydroxylating activity and a subsequent increase in peripheral levels of 17α-hydroxyprogesterone without a simultaneous rise in progesterone being apparent.

iv. The role of 17α-hydroxyprogesterone at mid-cycle

It has been established that a rise in 17α-hydroxyprogesterone occurs at mid-cycle in the normal menstrual cycle, and this rise possibly reflects either maturation or pre-ovulatory luteinization of a follicle. However, a role has not been assigned to 17α-hydroxyprogesterone. A possible role in gonadotrophin release must be considered.

Abraham et al., (1972) reported that in one subject an oestradiol peak occurred on day 9 of a 35 day cycle, but was not accompanied by an increase in LH. However, during the same cycle on day 22, an oestradiol peak of equal magnitude, concomitant with a significant rise in 17α-hydroxyprogesterone was then associated with an LH surge and a rise of progesterone in a luteal phase of 13 days. An analogous situation occurs in cycle 1 from subject KB (Fig. 4. VII) in the present study. This

suggests either that both oestradiol and 17α-hydroxyprogesterone are required to trigger LH release or that 17α-hydroxyprogesterone is merely reflecting follicular maturation. Abraham et al., (1972) also reported in a short luteal phase cycle, that an FSH rise was preceded by a peak of 17α-hydroxyprogesterone and an LH rise coincided with a peak of oestradiol. From this evidence they suggested a relationship between 17α-hydroxyprogesterone, oestradiol and gonadotrophin release and proposed the idea that 17α-hydroxyprogesterone may be related to FSH release and oestradiol to LH release. However, the present study does not support this hypothesis since increases in plasma levels of FSH are not always preceded by an increase in plasma levels of 17α-hydroxy-progesterone.

It appears from the present work that oestradiol is the most likely trigger of LH release and that 17α-hydroxyprogesterone is not an essential factor in gonadotrophin release. Oestradiol levels are always rising before LH begins to rise, whilst in many cycles 17α-hydroxyprogesterone levels rise only after the LH rise begins. This suggests that 17α-hydroxyprogesterone rises as a consequence of the LH rise, perhaps, as previously suggested, due to pre-ovulatory luteinization.

v. The Role of FSH in the Follicular Phase and at Mid-cycle

Evidence that FSH stimulates follicular growth is found in cycle 1 from subject KB (Fig. 4. VII). There appears to be a specific relationship between FSH and oestradiol in the follicular phase of this cycle. Discrete peaks of FSH are associated with peaks of oestradiol. It seems likely that low levels of oestradiol trigger FSH release which results in stimulation of the ovary and subsequent increased oestradiol production. It may be that in certain individuals the ovary is particularly sensitive to FSH stimulation and to 'bursts' of follicular growth. As the oestradiol production declines, release of FSH may again be stimulated until the growing follicle is sufficiently mature to maintain high levels of oestradiol over several days.

The role of FSH at mid-cycle has still to be elucidated. The mid-cycle peak of FSH is not always the major peak of the cycle. In subject EP (Fig. 4. X) an early follicular phase FSH peak is of equal magnitude to the mid-cycle peak and in cycle 2 from subject PH (Fig. 4. XIII) the FSH peak plateaus over six days. The FSH peak does not bear a consistent relationship in time to the LH peak. In some cases it falls on the same day (Subject PH, cycle 3, Fig. 4. XIV) whilst in others it falls

on the day after the LH peak (subject EP, Fig. 4. X) and in subject KB (cycle 1, Fig. 4. VII) it falls on the day before the LH peak. However, in all normal cycles where both FSH and LH results are available, the mid-cycle rises of the two gonadotrophins begin on the same day. The differences in patterns between LH and FSH seen in some cycles (e.g. Subject KB, cycle 1, Fig. 4. VII) suggest that the release of the two gonadotrophins are controlled separately although coincidently, at mid-cycle.

There is some controversy over the existence of one or more releasing factor. Dhariwal et al., (1965) claimed to have separated two distinct hypothalamic factors, one with LH releasing activity and another with FSH releasing activity. However, some workers now believe that only one releasing factor exists which triggers both LH and FSH release (e.g. Kastin et al., 1972). The present study is consistent with the view that either two separate releasing factors exist, which are perhaps produced due to stimulation by two separate ovarian factors, or that one factor exists which has both LH and FSH releasing activity and can release the two gonadotrophins separately depending on the stage of the cycle. Clearly these phenomena are extremely difficult to study in the human cycle where much interplay of

hormones is involved. Even direct measurement of the releasing factor(s) in peripheral plasma may be of limited use due to the very short half-life of the factor(s). Measurement of the factor(s) in hypothalamic vein samples may help to elucidate the problem, although this is an impractical task in the human.

Work by Rajaniemi and Vanha-Perttula (1972) may suggest a possible role for FSH at mid-cycle. Using autoradiographic studies they showed that, in rats, different ovarian receptors exist for $\left[{}^{125}\mathrm{I}\right]$ labelled gonadotrophins. LH was taken up by theca cells whilst FSH showed a preferential, though weak, accummulation in the granulosa cells of the developing follicle. This might suggest that LH and FSH are required for stimulation of different cell types at mid-cycle. However, this evidence is not consistent with the role of FSH in stimulating follicular growth by action on the theca cells resulting in increased production of oestradiol. The situation could, of course, be different at mid-cycle where the two gonadotrophins may stimulate luteinization of the different cell types. Also a species difference may exist between the rat and the human.

Kammerman et al., (1972) have shown, on the other hand, that porcine granulosa cells specifically bound HCG and that LH was able to compete for the HCG binding sites. They showed that

FSH had little binding capacity to these cells and concluded that it probably binds at different sites. These two pieces of evidence appear contradictory to some extent, but both indicate the possibility that the two gonadotrophins play different but specific roles at mid-cycle.

Another possible role of FSH at mid-cycle may be in 'priming' primordial follicles which may develop into Graafian follicles in future cycles. It is now well established that a single follicle grows to maturity not in one cycle, but over several cycles (Govan, 1973). Such follicles may require frequent stimulation by FSH and may produce oestradiol as a result of this. Their oestradiol production however may be masked by that of the corpus luteum. This hypothesis is purely speculative.

vi. The mid-cycle release of LH

There is now much evidence to suggest that oestradiol is the trigger of mid-cycle release of LH, both from work such as the present study, where the chronological sequence of events has been examined and by observing LH release after administering oestradiol (Yen and Tsai, 1972). From the results of the present study, which support those of other workers, neither progesterone nor 17α-hydroxyprogesterone can be implicated in this mechanism

as a rise in these hormones is not generally observed prior to a rise in LH. Monroe et al., (1972) have suggested that a decline in oestradiol from its peak levels could be responsible for the release but the present work shows that a decline in peak levels seldom if ever, precedes the first significant rise in LH.

In the normal cycle it is observed that mid-cycle peak levels of oestradiol are maintained over a number of days before LH is released. This suggests that the hypothalamus must be 'primed' in some way with oestradiol before LH release can be effected.

There appears to be no correlation between the peak height of oestradiol and the peak height of LH, i.e. - high mid-cycle oestradiol levels do not necessarily give rise to a high peak level of LH. This suggests that a threshold level must be reached (and sustained for several days) to bring about release, and secretion of oestradiol above this threshold does not ellicit further response. From the variation in oestradiol values found in the normal cycles, this threshold level appears to differ from one individual to another. It seems reasonable to postulate that this threshold is reached when the follicle is sufficiently mature to respond to LH stimulation. Peak

oestradiol levels vary greatly from cycle to cycle in the same individuals, whilst LH levels are relatively constant (e.g. subject PH, cycles 1, 2 and 3, Figs. 4. XII, XIII, and XIV). This suggests that this threshold level is unique for a particular cycle.

In discussing gonadotrophin levels it is important to remember that the peripheral levels of gonadotrophins are possibly only a reflection of that which is unbound to the follicular cells and as such, may not be an accurate measure of its physiological action. As the LH 'peak' is short-lived, once daily sampling is probably of little use in quantitative assessment of LH response. Much more frequent sampling over the 24 to 48 h period of the 'peak' must be undertaken to obtain a quantitative estimate of LH release.

In studying the mid-cycle period, most authors discuss the relationships between peaks in hormone levels when correlating events. Such relationships possibly have limited meaning when the variation in patterns seen in the individual cycles is considered. More useful information may have been obtained in the present study by relating the first significant rise of one hormone with respect to another, over the mid-cycle period.

vii. The role of LH at mid-cycle

A pre-ovulatory rise in progesterone is now fairly well documented (see p. 162). If this progesterone is a product of luteinization occurring before ovulation, the two processes may be dissociated. This raises the problem of the role of LH, whether it is a luteinizing factor, an ovulatory factor, or both. LH could conceivably bring about early luteinization of the follicle and this luteinization may, in turn, bring about changes resulting in ovulation. Full elucidation of the role of LH is beyond the scope of this study.

viii. Assessment of Corpus Luteum function

The patterns of the three steroid hormones, progesterone, 17α-hydroxyprogesterone and oestradiol are always similar in the luteal phase of the cycle. This suggests that the three hormones are produced by the corpus luteum and hence that they all reflect corpus luteum function. The luteal levels of the hormones however, vary greatly from cycle to cycle and there is no consistent relationship between relative levels of hormones (Table 4.2). High levels of one hormone are not always associated with high levels of either of the others. This might suggest that the hormones are produced by different cell types of the

corpus luteum, the cells having different secretory activity from one corpus luteum to another. However, as the pattern of hormones is always similar the different cell types appear to function as a whole. It is interesting to note that the length of the luteal phase appears to be independent of the amount of hormone produced (Table 4.2). Thus the peak hormone values and/or the area under the luteal phase curves may be more important in assessing corpus luteum function than the length of the luteal phase. There is good agreement, in general, between peak height and area under the curves for each hormone taken individually. This suggests that either of the two criteria could be a 'measure' of corpus luteum function in the normal cycle.

It is difficult to assess whether any one hormone alone could be used to 'measure' corpus luteum function. For example, if the cycles from subjects KK or KB (cycle 1, Figs. 4. IX and 4. VII), are examined, study of any one of the three steroids would be suggestive of an adequate corpus luteum. If, on the other hand, the cycle from subject MMc (Fig. 4. XI) is examined, progesterone levels would suggest an adequate corpus luteum but 17α-hydroxyprogesterone or oestradiol levels might suggest

a poor corpus luteum. It is difficult to decide therefore which hormone gives the true picture. Each of these subjects had an apparently normal cycle so it must be considered that in each case the levels observed were normal. Results in the normal cycles suggest that it is necessary to measure all three hormones to obtain a detailed picture, but measurement and observation of a suitable increase in any one hormone gives some evidence of luteal activity. Luteal phase increases in these hormones are generally used as indirect proof of ovulation having occurred. However, it must be considered that luteinization can occur without ovulation if the pre-ovulatory rise in progesterone discussed previously (p. 162) is produced by luteinized cells. Luteal phase increases in steroid hormones may thus provide evidence of luteinization but not necessarily of ovulation.

The BBT curve was biphasic in all normal cycles in which temperature was recorded. This increase in temperature corresponds to an increase in progesterone production by the corpus luteum and thus confirms that it may be a rough indicator of luteal function. It may not be an indicator of ovulation however, if luteinization, and thus increased progesterone production, can occur without ovulation.

The range of hormone levels found in the luteal phase in normal cycles is very large. Similar, or in some cases greater,

variation has been found by other workers (e.g. Mishell et al., 1971; Abraham et al., 1972; Thorneycroft et al., 1971). This great variation in levels of hormones leads to some difficulty in assessing corpus luteum function. It may suggest that the levels of hormones measured in peripheral plasma are perhaps not a true indication of the physiological state, although this measurement is the most practical which can be made at present.

The length of the luteal phase appears to be constant from one cycle to another in the same individual, even though the overall cycle length changes. This is noted in the three cycles from subject PH (Figs. 4. XII to 4. XIV). This is in agreement with many previous observations of the contancy of the luteal phase. The length of the follicular phase does not appear to be crucial. Cycles with short follicular phases do not necessarily show signs of inadequate follicular growth or inadequate corpus luteum function. The timing of the life-span of the corpus luteum appears therefore to be more critical than the period of follicular growth.

ix. <u>The use of single luteal phase progesterone estimations to assess corpus luteum function</u>

Israel et al., (1972) have suggested that a luteal phase progesterone level of 3 ng/ml or greater between days 11 and 4

prior to the onset of menses is presumptive evidence of ovulation. In the present study, several short luteal phase cycles (see p. 82) which appear to be ovulatory do not show this level by the 11th day before the onset of menses. The possibility of using 'spot' progesterone values as an indicator of ovulation must be viewed with caution, special attention being given to the time of sampling in relation to luteal phase length. Such 'spot' values are often suggested for use in clinical situations such as the monitoring of treatment for infertility. It has been found that patients treated with 'Pergonal' (Black et al., 1973) often reach these levels in apparently anovulatory cycles, so that 'spot' levels in such cases must be carefully evaluated. This practice is of little use when a detailed examination of hormone status is required. Used carefully, however, it may be useful when facilities are not available for assaying large numbers of samples.

B. 'ABNORMAL' CYCLES AND CONDITIONS

i. Selection of normal cycles

One of the major problems in presenting the results of a study, such as the present, is the definition of 'normal'. Based upon the broad criteria laid down (p. 65 - a luteal phase of

least 13 days and no known abnormality or special peculiarity) only 8 out of 19 cycles studied could be considered normal. This is not an uncommon finding - Newton et al., (1971) using similar criteria for normality rejected 5 out of 11 cycles studied. The only true indicators of ovulation are pregnancy or the isolation of an ovum following ovulation. Since the latter is impractical, the former must be regarded as the only true indicator. Only one of the subjects in the 'normal' group in the present study subsequently became pregnant and is perhaps the only one who should be categorised as definitely normal.

When two cycles from the same person were studied (e.g. subject PH, Figs. 4. XII and XIV) the results indicated that not only are patterns and levels of hormones different from individual to individual but also from one cycle to another in the same individual. Similarly patterns may differ from cycle to cycle even in subjects of proven fertility. Thus there would appear to be no suitable alternative to selecting normal cycles on the basis of clinically accepted criteria.

The normal cycles have been used in the present study to establish patterns and levels of hormones and to form hypotheses concerning the regulation of the cycle. Several of the 'abnormal' cycles also yield some interesting results concerning ovarian function.

ii. The short luteal phase cycles

The short luteal phase cycle must be carefully classified. In short luteal phase cycles described by Strott et al., (1970) and Abraham et al., (1972) it was not certain whether ovulation occurred or whether increased progesterone output in the 'luteal' phase was associated with luteinization of atretic follicles without ovulation. In several short luteal phase cycles examined, in the present study, hormone patterns and levels suggest normal ovulatory cycles (e.g. subject MA, Fig. 4. XV and subject CW, Fig. 4. XVIII). Johansson et al., (1971) also reported cycles with normal hormone levels but with luteal phases of less than 13 days. These results suggest that in this type of cycle there is no real basis for selecting a minimum of 13 days as being required for an adequate luteal phase.

Some of the short luteal phase cycles also showed other abnormalities. In cycle 1 from subject BW (Fig. 4. XVI) the oestradiol pattern in the luteal phase was abnormal, higher levels relative to the mid-cycle level were observed than in the normal cycle. Comparison of the two cycles (Fig. 4. XVI and 4. XVII) from this subject again shows that cycles may vary in the same subject. This is especially exemplified by the LH level at mid-cycle, the peak being twice as high in cycle 2 as

in cycle 1, whilst FSH patterns were similar in the two cycles. The mid-cycle level of oestradiol in the two cycles are of similar magnitude which again suggests that the peripheral level of oestradiol at mid-cycle is not related to the corresponding peripheral level of LH measured.

The two cycles from subject JG (Figs. 4. XIX and 4.XX) show wide variation in length of luteal phase. Cycle 2 (Fig. 4.XX) has a normal length (14 days) luteal phase whilst cycle 1 (Fig. 4. XIX) has only a 9 day luteal phase. Both cycles appear to have poor corpus luteum function on the basis of progesterone levels in the luteal phase. This suggests the hypothesis that length of luteal phase alone does not necessarily indicate a normal cycle.

An early transient rise in oestradiol in cycle 1 from subject JG (Fig. 4. XIX) is not associated with LH release. However, on day -2, a rise in oestradiol occurs which is again not sustained but is followed by release of LH and FSH. However, the luteal phase pattern of hormones suggests that a poor corpus luteum is produced, possibly by luteinization of an immature follicle. In this case, the ovarian/hypothalamic 'fail safe' threshold device may not have functioned and thus allowed LH to be released when the follicle was in an immature state, being unable to bind LH and produce an adequate corpus luteum. Thus the sustained

high level of oestradiol seen in a normal cycle and absent in this cycle, may well indicate follicular maturation. However, a rise in 17α-hydroxyprogesterone does not necessarily indicate follicular maturation as suggested by some authors (e.g. Strott et al., 1969). The source of the 17α-hydroxyprogesterone in this case is obscure.

iii. The effect of pregnancy on a subsequent cycle.

A cycle from subject KB (Fig. 4. XXIII) after termination of pregnancy when normal menstruation had been resumed, was studied. The results indicated some differences from the pre-pregnancy cycle (Fig. 4. VII). Cycle 2 had a short luteal phase and 'abnormal' steroid hormone patterns. As this is the only cycle studied from a subject following pregnancy it is difficult to draw conclusions. However, a more detailed study of this phenomenon is obviously required and in the meantime results from studies where cycles from both parous and non-parous women have been included in establishing hormone ranges, must be viewed in this light (e.g. Mishell et al., 1971; Johansson et al., 1971).

The pregnancy of this subject was terminated by infusion of prostaglandin E_2. As the half-life of prostaglandin is approximately five minutes it seems unlikely that this could still

be upsetting the cycle after the elapse of six months. However, some long term affect on the ovary cannot be ruled out. Prostaglandins are reported to be luteolytic in rhesus monkeys, as indicated by a reduction in plasma progesterone levels and the premature onset of menstruation (Kirton et al., 1970). A luteolytic effect has not been demonstrated by administration of prostaglandin in the luteal phase of the human menstrual cycle (Le Maire and Shapiro, 1972).

iv. The effect of an I.U.D. on the menstrual cycle.

There is some discrepancy as to the mode of action of the intra-uterine device in preventing pregnancy. Some authors suggest that its mode of action is purely by mechanical interference with implantation (e.g. Tietze, 1966) whilst others suggest that the device causes histological changes and effects hormone sensitivity of the endometrium (e.g. Bonney et al., 1966). Besides uterine effects, direct action on the ovary must be considered. Faucher et al., (1969) have shown that after insertion of an I.U.D. the luteal phase of the cycle was shorter and urinary pregnanediol output slightly reduced when compared with pre-I.U.D. values.

The cycle from subject SR (Fig. 4. XXII) who had an I.U.D.

present, suggests that even though the cycle had a short luteal phase (12 days) the I.U.D. had no dramatic effect on hormone levels or patterns. It is difficult to comment on the effect on length of luteal phase as a control cycle from this subject is not available. In the present cycle the high peripheral level of oestradiol observed, could conceivably reflect a decrease in the binding of oestradiol to endometrial tissue, thus indicating an effect on endometrial sensitivity. The present results, although from only one cycle, confirm the conclusions of Martin and Brown (1973) who suggest that the shortening of the luteal phase is not due to hormonal changes. These authors suggest that it is due to local effects of the I.U.D. which cause premature shedding of the endometrium.

v. <u>The effect of aspirin administration on luteal function.</u>

Subject HG (Fig. 4. XXIV) took large doses of aspirin during the luteal phase of this cycle. It seems unlikely that this had any effect on corpus luteum function. The hormone levels were low in the luteal phase but no changes appeared to be associated with the beginning of aspirin administration. The low hormone levels may however have been associated with the fact that the subject was unwell throughout the cycle which could have

had a psychosomatic effect on ovarian function.

vi. A possible anovulatory cycle

The cycle from subject DB (Fig. XXV) may exemplify the dissociation of ovulation and luteinization. The steroid hormone levels throughout this cycle are low and are not suggestive of a normal ovulatory cycle. The results of this cycle may be interpreted in the following way. The follicle does not respond adequately to FSH stimulation in the follicular phase, resulting in low production of oestradiol at mid-cycle. Nevertheless, LH and FSH release is triggered but the gonadotrophins are not adequately bound to the follicle as the cells are immature. This inadequate binding is reflected in high peripheral levels of LH and FSH. The steroid hormone levels are all below the normal range in the luteal phase which suggests that a 'poor' corpus luteum is present due to inadequate luteinization or that the steroids are produced by luteinized cells not necessarily associated with ovulation. Separate phenomena may be required to establish the occurrence of these two events in the menstrual cycle.

vii. A comment on abnormal cycles

Although more than half of the cycles studied in Chapter 4, have been classified as abnormal, it seems unlikely that so many subjects would prove infertile. This suggests that the present criteria for assessing normality are too strict. The difficulty in selecting normal cycles has been mentioned, but the necessity of establishing this normal group for comparison with infertile patients for clinical endocrinological reasons must be emphasised.

Much variation in patterns of hormones is seen in the abnormal cycles. This leads again to the conclusion that patterns of hormones in peripheral plasma are not necessarily a direct indicator of a physiological state. It may be misleading and impossible to interpret every hormonal change observed in peripheral plasma.

viii. Anorexia nervosa

The hormone patterns found during the period of anorexia nervosa in subject PH (Fig. 4. XXVI) are difficult to interpret. The high progesterone levels at the beginning of the sampling period may have been responsible for depression of gonadotrophin levels. However, the source of this progesterone is unclear. The cortisol levels do not suggest adrenal over-activity. The elevated progesterone levels were associated with moderately high

oestradiol levels and with low 17α-hydroxyprogesterone levels. Six days after the last blood sample was collected, the subject menstruated normally. The hormone patterns over the second half of this sampling period can therefore be explained as relatively normal cycle patterns. A peak of LH appeared on day 32, together with oestradiol and 17α-hydroxy-progesterone peaks. 'Luteal phase' rises in each steroid hormone occurred from day 34 onwards. However, the mechanism, by which the cycle was suddenly 'switched on' is difficult to establish, but could have been due to the drop in progesterone level on day 13.

PART III

A. HORMONE PATTERNS IN INFERTILE PATIENTS

The results presented in Chapter 5 indicate that there is great individual variation in the infertile patients. Comparison of the mean infertile cycles with the mean normal cycles (Figs. 5. VII to 5.XI) outlined the major differences between the two groups. However, in order to establish the possible cause of infertility in these patients, it may be useful to discuss each patient separately.

i. Individual Patients

Mrs. B. (Fig. 5. I. p. 97): Oestradiol levels in the follicular phase are indicative of poor follicular growth, but by mid-cycle the oestradiol peak suggests adequate maturation. A 'normal' mid-cycle LH peak suggests that the cycle is ovulatory and that luteinization should proceed normally (Abraham et al., 1972). Luteal phase levels of oestradiol and 17α-hydroxyprogesterone are within normal limits and show normal patterns. The luteal phase progesterone pattern however, is suggestive of a corpus luteum slightly retarded in growth, which takes a longer time to reach peak progesterone levels than does a normal corpus luteum.

The peak levels however, are within the normal ranges. The rise in 17α-hydroxyprogesterone prior to the LH peak coincides with a rise in progesterone. This may indicate pre-ovulatory luteinization as suggested in some of the normal cycles. In general the cycle appears to be ovulatory and does not have any major abnormality. It is therefore impossible to account for the infertility in this patient in terms of inadequate secretion of endocrine hormones.

Mrs. Mc (Fig. 5. IV. p. 102): In this patient, low follicular phase levels of FSH were found. Suppression of FSH secretion may have been due to the relatively high progesterone levels circulating in the early follicular phase. The low FSH levels however, did not appear to limit follicular growth as reflected in oestradiol production. A high mid-cycle level of oestradiol suggests adequate follicular maturation. No significant rise in the 17α-hydroxyprogesterone (or progesterone) occurred prior to the LH peak. This is further evidence that oestradiol and 17α-hydroxyprogesterone emanate from different cell types. A normal LH peak suggests that the cycle may be ovulatory. Although all steroid hormones reach normal levels in the luteal phase, they indicate retardation or, in the case of

oestradiol, fluctuations, in the development of the corpus luteum. This cycle raises the question of significance of the mid-cycle 17α-hydroxyprogesterone rise. Since such a rise is found in all normal cycles, but not in this infertile cycle, it should perhaps be considered as a marker of 'normal' ovarian function. The absence of a 17α-hydroxyprogesterone rise at mid-cycle is here associated with a possible reduced function of the corpus luteum, but not with reduced function of the growing follicle, since oestradiol secretion is normal. 17α-hydroxy-progesterone may perhaps be reflecting the first stage of luteinization and as such may be useful not as a marker of follicular maturation but as an early indication of subsequent luteinization. Alternatively, mid-cycle 17α-hydroxyprogesterone may be considered as a marker of follicular maturation by indicating that the growing follicle has reached a state in which it can respond to gonadotrophin stimulation.

No mid-cycle FSH peak is present which may indicate that FSH at mid-cycle is required for some process involved in steroid secretion by the corpus luteum.

Infertility may therefore be accounted for in this patient by retarded corpus luteum growth. It is difficult to explain the reason for this phenomenon especially as there is no indication of poor follicular growth.

Mrs. C. (Fig. 5. III. p.100): Low follicular phase levels of oestradiol presumably in response to low FSH levels were observed. It is not possible to comment on the mid-cycle level of oestradiol as the sample which was lost was likely to have been the mid-cycle peak sample. This result would presumably have been low compared to the normal cycle. However, very high levels of FSH and LH occurred at mid-cycle. Steroid hormone patterns in the luteal phase suggest formation of a poor corpus luteum which takes a longer time to reach peak steroid production than does a normal corpus luteum. The high peripheral gonadotrophin levels may indicate low binding of gonadotrophin to the ovarian cells resulting in poor luteinization. A rise in 17∝-hydroxy-progesterone occurs at mid-cycle but it is not accompanied by a rise in progesterone. The results in this cycle suggest that events occurring in the follicle before ovulation have much bearing on the 'quality' of corpus luteum formed. A follicle which does not grow efficiently will not luteinize efficiently. This suggests that the follicular cells must reach a certain state of maturity before they are able to bind gonadotrophin and form an adequate corpus luteum.

Mrs. T. (Fig. 5. V. p. 103): Despite normal FSH levels in the follicular phase, oestradiol levels are very low,

probably indicating poor follicular growth. In this case, the cells of the follicle do not appear to respond to FSH stimulation. Levels of oestradiol are low at mid-cycle, but a normal rise in 17α-hydroxyprogesterone occurs which is not accompanied by a rise in progesterone. High mid-cycle LH levels may again reflect low binding of LH to immature follicular cells. However, in spite of poor follicular growth, steroid levels in the luteal phase are within the normal range. The mid-cycle 17α-hydroxy-progesterone peak may again be signifying the first stages of luteinization rather than maximum follicular growth. There are signs that the corpus luteum takes longer to reach maturity than a normal corpus luteum.

Mrs. R. (Fig. 5. II. p. 99): FSH and oestradiol levels are low in the early follicular phase. However, by mid-cycle oestradiol levels are high. No rise in 17α-hydroxyprogesterone occurs at mid-cycle again suggesting that oestradiol and 17α-hydroxyprogesterone emanate from different cell types. Although the significance of this 17α-hydroxyprogesterone peak is not clear, its absence is again associated with an 'infertile' cycle. If 17α-hydroxyprogesterone is an early indicator of luteinization, absence of this peak may be expected to indicate retarded luteal growth. Although the steroid hormones fall within the normal range in the luteal phase, a retarded rate of growth is apparent.

It is interesting to note that in this cycle, as in Mrs. Mc's cycle, relatively high progesterone levels in the early follicular phase may have an inhibiting effect on levels of FSH. High early follicular phase levels of FSH may be required to stimulate the follicle early in its growth period. In these patients, the pituitary threshold for LH release may possibly be too low so that release occurs before the follicle is sufficiently mature.

Mrs. O. (Fig. 5. VI. p. 105): This cycle appears to be anovulatory. FSH levels are normal in the early follicular phase which suggests that either FSH may not be the only factor essential for the follicular growth or that the cells of the ovary are unable to respond to FSH stimulation. No mid-cycle rise in oestradiol is seen and hence no LH peak. However LH levels rise gradually throughout the cycle. The reason for this rise is difficult to explain. It is also difficult to account for menstruation in this cycle on the basis of traditional concept of oestrogen and progesterone withdrawal. This may suggest that the levels of these hormones measured in peripheral plasma are not an accurate indicator of levels affecting the endometrium. It may also indicate that in some

individuals the endometrium is extremely sensitive to hormonal influences and that menstruation can be associated with very small changes in hormone levels which are perhaps too small to measure in peripheral plasma.

The occurrence of menstruation in this cycle may, on the other hand, indicate that there is some other control mechanism functioning in the menstrual cycle besides the hormone relationships which have been examined in the present study. Prostaglandins for example, may be concerned in menstruation. Pickles et al., (1965) have identified prostaglandins in the endometrium and menstrual fluid in normal women.

ii. General Reasons for Infertility

It is difficult to draw general conclusions about the cause of infertility. The problems seem to lie, in some cases, in inadequate follicular growth which is accounted for either by low FSH levels or by a poor response to FSH by the follicular cells. It is apparent that the events of the follicular phase predetermine the luteal phase to some extent.

In the normal cycle, a sustained rise in oestradiol occurs at mid-cycle, and the maintenance of peak levels for several days appears to be required to produce LH release. This sustained rise is absent in some of these infertile patients.

This may suggest that there is a low threshold of LH release in these patients which allows release of LH before the follicle is mature. This may lead to luteinization possibly without ovulation, if the ovum is under-developed. In some cases high peripheral LH levels at mid-cycle may reflect poor binding of the LH to cells which are not sufficiently mature.

In some cases an inadequate corpus luteum with a retarded rate of growth may be formed by luteinization of a poorly grown follicle.

The reason for the poor follicular growth may lie in the selection of the particular follicle which matures in each cycle. It is well documented in the human that several follicles grow to a certain stage of development but only one is selected each month to grow to maturity. The mechanism of this selection is unknown but presumably, this particular follicle has some special characteristic. This follicle may be the only one capable of successful maturation and ovum fertilization. Conceivably, this 'selection' mechanism may not function in these patients and the 'wrong' follicle may grow and in some cases appears to reach maturity but is not capable of normal function. This abnormality may be concerned with the ovum, which might account for the comparatively normal hormone patterns which are sometimes found in cycles from these patients.

Such a suggestion cannot be tested until the mechanism of follicle selection is elucidated.

iii. Assessment of Corpus Luteum Function in Infertile Patients

The comparison of hormone peak heights to areas under the luteal curves (Table 5. 1.) indicates that there is good correlation in these patients between peak height and area; hence the higher the peak height, the greater the area under the curve. This evidence does not support Newton's (1973) idea that the total area is a more useful measure of corpus luteum function than peak height, but is of course, only valid in this particular group of patients. As in the normal cycles, there is little correlation between peak height or area for one hormone and those for another. It is difficult to decide which hormone is the most useful measure of corpus luteum function. Indeed each must be measured to obtain a complete picture of luteal phase in these patients.

It is interesting to note, although difficult to explain, that levels of 17α-hydroxyprogesterone often appear normal whilst other hormones show abnormality in pattern and level in the luteal phase.

All of the infertile patients have 'normal' length luteal phases. This suggests that the length of the luteal phase cannot be used as a criterion for the adequacy of the corpus

luteum. The follicular phases also are of 'normal' length but this is not necessarily associated with adequate follicular growth.

iv. Correlation of Hormonal and Morphological Changes

The clinical reports on several of these patients suggest that much variation occurs from cycle to cycle. For example, the biopsy report on Mrs. B. (Fig. 5. I. p. 97) suggested that ovulation had not occurred recently prior to the cycle of study. It is surprising therefore, that the cycle studied was apparently ovulatory. This suggests that either ovulatory cycles occur randomly and infrequently in this patient or that the cycle which showed presumptive signs of ovulation was in fact, anovulatory. There was no direct evidence of ovulation - a corpus luteum was not observed. It is possible that hormone patterns in such a cycle result from follicular growth with subsequent oestradiol production, LH release and the formation of a secretory body indistinguishable from a normal corpus luteum formed by luteinization of follicular cells, without ovulation. If this were the case, LH would be implicated as a luteinizing rather than an ovulatory factor. The apparently normal output of progesterone and oestradiol would account for

the presence of a secretory endometrium and the temporal pattern of the cycle. Previous suggestions that the LH peak may be a presumptive marker of an ovulatory cycle (Abraham et al., 1972) must thus be viewed with caution.

Biopsy reports on the other patients suggested in general, normal ovarian morphology. There is therefore, no reason to suspect anovulatory cycles. However, in the one patient who was almost certainly anovulatory in the cycle studied, Mrs. O. (Fig. 5. VI. p. 105), biopsy revealed a corpus luteum, indicating previous recent ovulation. It appears therefore that in this patient variation occurred from one cycle to another. If, in some of these patients, ovulation does not occur in every cycle, this will obviously limit their fertility. It may be difficult to differentiate therefore between an infertile and a sub-fertile condition.

b. EFFECTS OF 'CLOMID' TREATMENT

i. Mode of Action

Hormone patterns during cycles in which 'Clomid' was administered were compared to the pre-treatment cycles and to the normal cycle in Chapter 6.

In general, treatment with 'Clomid' is only recommended in cases of anovulation (Bishop, 1970). However, a close examination

of its possible mode of action in patients in this study suggests that it may be useful in their treatment.

'Clomid' is thought to act either by stimulation at the hypothalamus-pituitary level or at the ovarian level. Jacobson et al., (1968) and Newton and Dixon (1971) have induced changes in blood concentration of LH and FSH which were similar to those found in the normal cycle. Smith (1966) and Hammerstein (1969) have shown increased output of oestrogens on treatment, suggesting a direct ovarian effect. An oustanding feature of the present results was the wide variation in response to 'Clomid'. Such variation was also noted by Adamapoulos et al., (1972). It is difficult to assess whether 'Clomid' has any direct effect on FSH levels. The increase in early follicular FSH levels, in subjects Mrs. Mc (Fig. 6. VI) and Mrs. T. (Fig. 6. IV) cannot be accounted for by 'Clomid' treatment as the levels were elevated before treatment was begun. The results may suggest that these patients normally differ greatly from cycle to cycle in their FSH patterns. Even in Mrs. O., (Fig. 6. VII. p. 122) who became pregnant, no increase in levels over the pre-treatment cycle was seen. However, in this patient, the sharp drop in FSH levels in the pre-treatment cycle, was modified to a more gentle decline in the treatment cycle. In only one patient, Mrs. C. (Fig. 6. III. p. 116) was a rise in FSH associated with 'Clomid' treatment and in this case, treatment was administered

from day 6 to day 10. The results do not suggest that 'Clomid' elevates FSH levels in the early follicular phase.

'Clomid' does appear to have a direct effect on oestrogen production. It was conceivable that the increased oestradiol levels are an artefact due to cross reaction of 'Clomid' with the oestradiol antiserum. However, Table 3.5. indicates that 'Clomid' has no cross reaction with the antiserum used in the RIA. In all patients, except Mrs. R. (Fig. 6. V. p. 119) treatment with 50 mg of 'Clomid' increased the production of oestradiol. The mid-cycle peak height was elevated in most cases, but not in every case. However, in all cases the high levels were maintained for a longer time than in the pre-treatment cycles and appeared similar to the normal cycles. It is interesting that one patient (Mrs. R.) did not respond to treatment with 50 mg of 'Clomid'. However, this patient was treated from day 6 to day 10 of the cycle. In this individual, the ovary may not have been responsive to this dose or to the later starting time. The effects appear to be very individual since when Mrs. C. (Fig. 6. III. p. 116) was treated from days 6 to 10, the ovarian response was as great as that seen in any of the other patients.

Corresponding increases in mid-cycle levels of either LH or FSH are not generally associated with the elevated oestradiol levels. In only one subject, Mrs. B. (Fig. 6. II. p. 115) was there an elevation in LH but this could be due to the normal variation which might be expected from one cycle to another in the same individual. The mid-cycle FSH level is considerably lower in Mrs. C. (Fig. 6. III. p. 116) after treatment with 'Clomid'. Mid-cycle FSH levels appear to be elevated in Mrs. Mc. (Fig. 6. VI. p. 120) and Mrs. T. (Fig. 6. IV. p. 118), after treatment.

A second major effect of 'Clomid' appears to be on luteal phase levels of steroid hormones. In most cases the luteal phase progesterone levels are vastly increased after 'Clomid' treatment, often to above normal levels. However, in some patients major luteal phase increases in oestradiol and 17α-hydroxyprogesterone do not accompany the progesterone increases (e.g. Mrs. B., Fig. 6. II. p. 115 , and Mrs. T., Fig. 6. IV. p. 118). This may suggest that 'Clomid' is altering the metabolic capabilities of luteinized cells. The increases in progesterone may suggest either the formation of a 'better' corpus luteum or the presence of more than one corpus luteum. In view of the high progesterone levels reached, the latter possibility cannot be excluded. In Mrs. C. (Fig. 6. III. p. 116) for example, the results could depict stimulation of the

ovary resulting in the maturation of more than one follicle, reflected in the high mid-cycle oestradiol output. Perhaps none of these follicles properly reach maturity but luteinization of several follicles result in the presence of several corpora lutea. If the luteal progesterone pattern signifies multiple ovulation, the drug must obviously be used with care to avoid multiple pregnancies. It is difficult to assess whether the increased luteal activity is directly due to 'Clomid' or whether it merely arises from previous follicular stimulation. Herrman (1963) suggested that a direct effect on the corpus luteum could occur as he produced further increases in pregnanediol excretion by starting administration after ovulation. The metabolic half-life of 'Clomid' in the human body when administrered orally, is five days (Merrell-National Labs. Ltd., 1966). It seems unlikely therefore, that when given from days 1 to 5 of the cycle, there would be sufficient remaining in the body in the luteal phase to have a direct effect on the corpus luteum. As the luteal phase progesterone increases are always associated with increased mid-cycle secretion of oestradiol, it seems more likely that increased luteal activity results from increased follicular growth. In some cases, peak luteal phase progesterone levels were reached earlier in 'Clomid' treatment cycles than

in the pre-treatment cycle, but the peak levels were often short-lived (e.g. Mrs. T., Fig. 6. IV. p. 118). Lengthening of the luteal phase was not observed on treatment.

In several treatment cycles a pre-LH peak rise in progesterone was observed which corresponded to simultaneous increases in 17α-hydroxyprogesterone (e.g. Mrs. B., Fig. 6. II, p. 115 ; Mrs. C. Fig. 6. III, p. 116 ; Mrs. R., Cycle 2, Fig. 6. Vc, p. 119). These pre-ovulatory rises in progestogen could signify early luteinization of pre-ovulatory follicles. Major changes in 17α-hydroxyprogesterone levels are not generally induced on 'Clomid' treatment. A rise prior to the LH peak was induced in Mrs. Mc. (Fig. 6. VI, p. 120) and in all cases pre-ovulatory rises in 17α-hydroxyprogesterone in treatment cycles did not occur until well after the oestradiol rise was established. 17α-hydroxyprogesterone does not appear to immediately reflect, follicular growth.

Thus the mode of action of 'Clomid' appears, from the results of the present study, to be directed at the ovarian level. There is little evidence for action on gonadotrophin levels either directly or by prior ovarian stimulation. Jaffe and Midgeley (1970) reported that when given to normally menstruating women on days 5, 6, and 7 of the cycle, no consistent differences in gonadotrophin pattern could be found between that, and the

control cycle. An effect on the luteal phase is generally observed but the present results do not indicate whether this effect is direct or indirect. The possibility of multiple ovulations cannot be ruled out.

ii. Success of 'Clomid' Treatment

The only true indicator of the usefulness of 'Clomid' treatment in these infertile patients would be the occurrence of pregnancy. It is perhaps very significant that the only patient to become pregnant in this study was the anovulatory patient. Hence this may indicate that 'Clomid' is only useful in treatment of anovulatory infertility. Each of the other patients have now undergone several courses of 'Clomid' therapy without success. At the present time this is the only well established treatment available for these patients. Preliminary trials have been carried out in women by Klopper and Hall (1971), using the compound ICI 46474 (p-β-dimethylamino-ethoxyphenyl-1,2-diphenylbut-1- ene) for the treatment of infertility and have found it to be successful in inducing ovulation. However, this compound has not had such widespread use as 'Clomid'.

The results indicate much variability in individual response to 'Clomid' and suggest that each patient must be treated indiv-

idually and carefully monitored during treatment. Sufficient results are not available to decide if one treatment regime is better than another. It is unlikely that increasing the dosage would be useful in these patients. This may simply produce greater risk of multiple ovulations.

Even though pregnancies have not resulted, there is evidence to suggest that the cycles have been improved by 'Clomid' therapy as indicated by hormone secretion both in follicular and luteal phases, though in some cases levels of hormones above the normal ranges have resulted. This is perhaps, sufficient indication that 'Clomid' treatment should be attempted in patients such as these.

In the present study, clomiphene citrate which is a 1:1 mixture of cisclomiphene and transclomiphene has been employed. Van Campenhout et al., (1973) reported that cisclomiphene was more potent than the trans-isomer for induction of ovulation. It would be interesting to compare the effect and thus the mode of action, of the cis-isomer in these patients.

PART IV

a. GENERAL COMMENTS

There are many factors which must be taken into consideration when interpreting results from a study such as this. One of the most important of these may be the possible effect of daily vene-puncture in these subjects. Johansson et al., (1971) suggested that daily vene-punctures may be a profound stress factor in women not familiar with hospital routine and that this factor may effect hormonal events during the menstrual cycle.

Another factor which must be considered is the number of subjects included in the study. A large proportion of the 'normal' volunteer subjects had to be rejected from the normal series so that the final number on which mean levels and ranges were based was small. In view of the variation observed, as many subjects as possible must be included in such a study. The number of infertile patients studied was also small. It is no doubt necessary to study many more patients who fit into this category in order to build up a complete picture of the condition.

One criticism of the work may be that the samples have not been assayed in duplicate. This decision was not taken

without careful consideration but it was decided that a greater error might be introduced by assaying samples from a cycle in two assays rather than in the same assay. It would have been impractical to measure in duplicate, samples from a complete cycle, together with blanks and quality control samples in a single assay.

The gonadotrophin results were obtained in other laboratories. It is therefore difficult to criticise or comment on these. Some of the FSH results were difficult to interpret. However, it is easier to criticise ones own results than those of others.

Although subtle differences have been found between the normal and infertile patients, no gross abnormality in the hormones measured accounts for their infertility. It is possible that in these patients some other factors must be examined which might account for their condition. Psychological factors might be expected to have their effect through the hypothalamic-pituitary axis, causing inhibition of ovulation. Since these patients appear to ovulate such factors can probably be ruled out.

It is possible that other hormones must be studied in these patients to complete the picture. Conceivably abnormalities in androgen patterns could account for abnormal ovarian function.

Androgens have been shown to affect ovarian function in rats (Fels and Bosch, 1971). Advances in RIA have made androgen estimation throughout the menstrual cycle possible (Judd and Yen, 1973). Androgen levels must be established in normal women and in infertile women and at the same time the possible role of androgens in female reproductive physiology must be investigated.

The abnormalities in these infertile women may, on the other hand, not be hormonal. The possible formation of antibodies to their husband's sperm (e.g. Franklin and Dukes, 1964; Pacheco-Romero et al., 1973) has not been eliminated nor has the possibility of some abnormality in the husband, in spite of normal sperm counts.

Whilst hormone patterns have been related to expected morphological changes, these changes have not been physically monitored in either normal or infertile subjects. Ovarian and endometrial biopsies were performed in infertile patients prior to study but not in the cycle of study. Whilst increased levels of hormones in the luteal phase of the cycles has been associated with a corpus luteum, the presence of corpora lutea have never been visually established. There is no direct evidence that any of the cycles studied were ovulatory except in the one subject who became pregnant.

Studies of this type on the human obviously have some short-comings. However, these are not easily overcome. Sampling of ovarian vein blood may provide more useful results than peripheral sampling but this would be clinically impractical in the human. This type of study is perhaps the best which can be carried out in humans in vivo.

b. SUMMARY OF RESULTS

The results and conclusions reached in this study are summarised with reference to the aims of the work set out in Chapter 1 (p. 18).

1. Accurate, precise and reliable methods have been developed and evaluated for the measurement of progesterone, 17α-hydroxyprogesterone and oestradiol in human plasma. These methods are suitable for routine use in the laboratory and give results comparable to those of other workers.

2. Ovarian and pituitary function has been studied in a group of 'normal' volunteer subjects. These studies have yielded much information about the normal menstrual cycle and also about cycles which could not be considered normal by the definition accepted for the purposes of this study (p. 65). Normal ranges for each hormone have been established.

3. Hormone patterns and levels have been studied in a selected group of infertile women and compared to the normal group. The results indicate that subtle differences exist between the normal and the infertile which could account for the infertility. Poor follicular growth and maturation followed by the formation of an inadequate corpus luteum may explain the condition.

4. Treatment of these patients with 'Clomid' has resulted in better follicular growth and apparently improved corpus luteum function. However, it is undecided whether the increases in hormone levels represent 'better' growth of one follicle, followed by the formation of a 'better' corpus luteum or whether morethan one ovulation is involved. No dramatic effect on gonadotrophin levels was detected. Hormone levels in the luteal phase were frequently elevated above normal levels in 'Clomid' treatment cycles.

5. In these patients it is concluded that the mode of action of 'Clomid' lies at the ovarian rather than the hypothalamic-pituitary level. 'Clomid' appears, in general, to be a useful form of treatment in these patients in so

far as improved follicular and corpus luteum function is concerned. However, the treatment has not succeeded in stimulating pregnancies (except in one case).

6. The use of measurement of oestradiol, progesterone and 17α-hydroxyprogesterone as markers of presumptive morphological changes has been assessed. Oestradiol reflects follicular growth as an increase in the levels of this hormone precede ovulation of a mature follicle. The mid-cycle rise in 17α-hydroxyprogesterone may reflect follicular maturations as this rise in 17α-hydroxyprogesterone frequently precedes ovulation. However, this peak appears more likely to be a marker of pre-ovulatory luteinization of a mature follicle rather than a reflection of increased oestradiol production.

   All three steroid hormones appear to reflect corpus luteum function. An increase in all three is associated with presumptive formation of a corpus luteum. However, all three should be measured to accurately assess corpus luteum function especially in the 'Clomid' treated patient.

c. FUTURE WORK

Several problems concerning the regulation of the human menstrual cycle remain to be elucidated. The major ones are :-

The Source and role of mid-cycle 17α-hydroxyprogesterone.

The role of mid-cycle FSH.

The mechanism of luteinization and ovulation.

The mechanism of follicle 'selection' in the menstrual cycle.

The role of the oocyte in ovarian function.

Metabolic studies on isolated ovarian cells seems an obvious continuation of this project. Some work in the human has been reported (e.g. Ryan and Petro, 1966; Ryan et al., 1968) in which granulosa and theca cells have been separated and their metabolism studied by incubation with $\left[^{14}C\right]$ steroid precursors in vitro. However, in these previous studies with human follicles, the patients have been pre-treated with 'Pergonal' before the ovaries were removed. Channing et al., (1968) report that such treatment may cause alterations in steroid production. Work is at present in progress in this laboratory to separate granulosa and theca cells from normal, untreated human ovaries at various stages of the cycle and to incubate them in vitro, with various precursors. This study will be aimed initially, at investigation of the

hypothesis concerning the source of mid-cycle 17α-hydroxyprogesterone, by study of the predominant pathways in the two cell types. It is envisaged that such *in vitro* systems will be useful in studying binding of gonadotrophins to different cell types in follicles at various stages of development. This may lead to elucidation of the role of FSH at mid-cycle and to a fuller understanding of the luteinization process. However, it must be borne in mind in such studies that the demonstration of the capacity of a cell to synthesize a substance *in vitro*, or of a process to occur *in vitro* cannot be used as proof of the occurrence of that same synthesis or process *in vivo*.

A fuller understanding of the regulation of the normal cycle must be achieved before the condition of infertility can be explained.

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APPENDIX

CALCULATION OF RESULTS IN THE COMPETITIVE PROTEIN BINDING ASSAY

CALCULATION OF RESULTS IN THE COMPETITIVE PROTEIN BINDING ASSAY

CALCULATION OF $\left[^3H\right]$ INTERNAL STANDARD RECOVERED

If x is the total dpm of $\left[^3H\right]$ steroid added as internal standard and y is the dpm in a 0.2 ml aliquot from 1 ml of eluate, the % recovery of $\left[^3H\right]$ steroid will be :-

$$\frac{y}{1/5\ x} \times 100\ \% = z\ \%$$

Calculation of % $\left[^3H\right]$ bound in the CPB assay

If, following dextran coated charcoal precipitation, a is the mean dpm contained in an aliquot of supernatant in the 'No charcoal' tubes, and b dpm are contained in an aliquot of supernatant from the sample assayed, the % $\left[^3H\right]$ bound in that sample will be:

$$\frac{b}{a} \times 100\ \%$$

STANDARD CURVE

The dpm in an aliquot of supernatant from each sample comprising the standard curve is calculated as a percentage of the mean dpm in an aliquot of supernatant from the 'No charcoal' tubes. The resulting % $\left[^3H\right]$ bound is plotted against mass of 'cold' steroid in each sample.

CORRECTION FOR EXCESS $\left[^3H\right]$ ADDED AS INTERNAL STANDARD

Since the dpm originating from added $\left[^3H\right]$ tracer in 1/5th of the final eluate is y , the excess dpm present in the remaining 4/5 of the sample which was subjected to CPB assay will be :-

$$y \times 4 \times \% \quad \text{bound}$$

$$\therefore \; y \times 4 \times \frac{b}{a} = \text{excess dpm/aliquot of supernatant}$$

since only b/a % of the excess dpm were bound.

The corrected dpm is then obtained by subtraction of the excess dpm from the total dpm (b), in the aliquot of the supernatant of the sample.

$$\text{i.e. corrected dpm} = b - \left(y \times 4 \times \frac{b}{a}\right)$$

This corrected dpm is then used to calculate a new % $\left[^3H\right]$ bound:-

$$\frac{b - \left(y \times 4 \times \frac{b}{a}\right) \times 100\ \%}{a}$$

FINAL RESULT

The mass of steroid, c, corresponding to the % $\left[^3H\right]$ bound calculated above is obtained from the standard curve. Correction is then made for procedural losses.

Since only 4/5 of the total sample was assayed and z was the % of sample recovered throughout the purification step :-

$$\text{mass in sample} = \frac{c \times 100}{0{\cdot}8 \times z} \quad \text{ng}$$

A final correction is made, where necessary, to take into account the volume of plasma used in the assay, the final result being expressed in ng/ml plasma.

PUBLICATIONS

PUBLICATIONS

The following communications have been published or accepted for publication during the course of this work.

Dodson, K. and Coutts, J.R.T. (1972)
17α-hydroxyprogesterone levels as a measure of corpus luteum function.
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Copies of the communications which have been published are inserted in the back cover of the thesis.