The influence of progesterone and oestradiol on migraine

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THE INFLUENCE OF PROGESTERONE AND OESTRADIOL ON MIGRAINE

by

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School of Medicine,
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FOREWORD

This thesis contains the work of the author while Sandoz Research Fellow and Commonwealth Postgraduate Scholar in the Division of Neurology, School of Medicine, University of New South Wales. The following papers arising from this work have been published or accepted for publication:

Somerville, B.W.: "The Role of Progesterone in Menstrual Migraine". Neurology (Minneapolis): In press.

Somerville, B.W.: "The Role of Estradiol Withdrawal in the Etiology of Menstrual Migraine". Neurology (Minneapolis): In press.

Somerville, B.W.: "Daily Variations in Plasma Levels of Estradiol and Progesterone during the Normal Menstrual Cycle". American Journal of Obstetrics and Gynecology: In press.

Somerville, B.W. and Carey H.M.: "The Use of Continuous Progestogen Contraception in the Treatment of Migraine". Medical Journal of Australia, 1: 1043 (1970).

Lance, J.W., Anthony, M. and Somerville, B.W.: "Comparative Trial of Serotonin Antagonists in the Management of Migraine". Brit. Med. J., 2, 327 (1970).

Lance, J.W., Anthony, M., and Somerville, B.W.: "Thermographic, Hormonal and Clinical Studies in Migraine". Headache, 10, 93 (1970).

Somerville, B.W.: "The Influence of Hormonal Changes Upon Migraine in Women". Proc. Aust. Ass. Neurol.: In press

The following paper has been submitted for publication in Neurology (Minneapolis):

Somerville, B.W.: "A Study of Migraine in Pregnancy"

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INTRODUCTION

"Haec singulis mensibus, saepius instante mensium fluxu, interdum cessante, corripitur singulari capitis dolore, hemicrania, circa tempora, nunc in dextra nunc in sinistra capitis parte, haerente & lancinante.

Invadit hic dolor cum frigore aliquo & horrore, nauseaque & ventriculi dolore. Quae symptomata quando ambulando vincere conatur, augentur, si lecto se committit, nonnihil minuuntur, excepta nausea, cum fluore copiosi serosi humoris ex ore, qui non prius cessat, quam dolores remittunt, quod plerumque elapso spatio viginti & quatuor horarum accidit, posthac instar sanae surgit & comedit more solito".

Johannis Antonidae van der Linden,
in "De Hemicrania Menstrua" (1666).

Physicians have long observed that in some women, migraine may recur regularly with menstruation. Thus, Johannis van der Linden, describing migraine in the Marchioness of Brandenburg, mentions that she suffered from a severe

unilateral headache, accompanied by nausea and vomiting, lasting for 24 hours, and that this headache recurred each month "during the menstrual flux". Apart from the existence of menstrual migraine, several other clinical observations point to the significance of changes in female sex hormones in influencing migraine.

For example, pregnancy usually causes a remission of migraine (Friedman and Merritt, 1959). Relief from migraine during pregnancy is more likely to occur if the attacks have a menstrual periodicity (Lance and Anthony, 1966). Following the widespread use of synthetic oestrogen-progestogen combinations for the suppression of ovulation, several reports have described an aggravation of pre-existing migraine, or the appearance of migraine de novo in women taking these hormones. There is now general agreement that a causal relationship exists between the taking of oestrogen-progestogen combinations and the exacerbation of migraine.

The investigation of the influence of female sex hormones upon migraine evidently depends upon the accurate quantitative measurement of these hormones in the body. Earlier methods of hormone assay, based on measurement of the urinary excret-ion of hormone metabolites, would be unsatisfactory, since these methods provide only a delayed picture of the pattern of circulating hormones, and in addition, introduce several

important variables, such as hepatic detoxication and renal function. It is clear that there is a need for a technique for the direct measurement of sex hormones in blood, prefer--ably requiring only small samples of blood, so that serial (e.g. daily) hormone assays could be performed.

These requirements have been met by using the principle of competitive protein-binding, first adapted to the measure--ment of steroid hormones in plasma by Murphy, Engleberg and Pattee (1963). Thus, in the studies reported later in this thesis, it has been possible to measure progesterone accurately in plasma samples of 0.25-1.0 ml, and to measure oestradiol in samples of 4 ml. The small volume of plasma required has enabled the daily measurement of plasma levels of both these major female sex hormones to be made throughout the menstrual cycle.

The principal part of this thesis concerns the investigation of hormone patterns in women suffering from menstrual migraine, and in normal, non-migrainous women. The role of each of the two major sex hormones was assessed by treatment of migrainous women with progesterone, then with oestradiol. Conclusions are then drawn regarding the contribution of each to the precipitation of menstrual migraine.

In view of the reported beneficial effect of synthetic progestogenic hormones in migraine (Lundberg, 1962), a small pilot trial involving the continuous administration of a synthetic progestogen, 3-acetoxychlormadinone (AY 11440) was undertaken. Observations arising from this trial are considered in relation to the possible mechanism of migraine.

The effect of pregnancy upon migraine was then studied, in order to resolve the conflict between the traditional view that pregnancy has a generally beneficial effect upon migraine, and the more recent findings reported by Callaghan (1968), which suggest that migraine may be adversely affected by pregnancy. This investigation was concerned both with biochemical and clinical aspects of migraine during pregnancy, and involved the questioning of 200 women attending the antenatal outpatients' clinic at the Women's Hospital, Crown Street, Sydney.

Finally, the results of these investigations have been integrated into a hypothesis concerning the role of female sex hormones in the aetiology of migraine.

CHAPTER 1

THE MENSTRUAL CYCLE

1. THE MENSTRUAL CYCLE

Introduction

Since the major part of this work concerns the mechanism of migraine occurring in association with menstruation, it will be necessary to begin by reviewing current knowledge of the physiology of the menstrual cycle. Information concerning the hormonal events which occur during the menstrual cycle has undergone spectacular expansion in recent years. This has been largely the result of the development of techniques of assaying hormones in very small samples of blood. The contribution of competitive protein-binding techniques has already been mentioned. Another useful principle has been that of radioimmunoassay, which has been applied successfully to the measurement of the gonadotrophic hormones in plasma. Thus, radioimmunoassay of Luteinising Hormone (LH) has been described by Midgley and Jaffe (1966) and Odell, Rayford and Ross (1966). A similar technique for the measurement of Follicle-Stimulating Hormone (FSH) in plasma has been described by the same authors (Odell, Ross and Rayford, 1967).

These technical advances have made possible the simultaneous measurement of LH, FSH, oestradiol and progesterone of plasma daily throughout the entire menstrual cycle. In this way, it has been possible to study the interrelationships between

pituitary gonadotrophins and ovarian hormones. The relation-ship between plasma levels of LH and progesterone have been studied by Neill, Johansson, Datta and Knobil (1967); Cargille, Ross and Yoshimi (1969) have investigated plasma levels of LH, FSH and progesterone during the cycle; and Corker, Naftolin and Exley (1969) have measured plasma levels of oestradiol and LH in attempting to clarify pituitary-ovarian hormonal interrelationships.

In the following review, emphasis will be placed upon the hormonal events of the menstrual cycle. Structural changes within the ovaries and uterus will be considered briefly. The numerous extragenital physiological changes which occur during the cycle will receive only brief mention.

The Role of the Hypothalamus and Hypophysis

The hypothalamus plays a key role in the regulation of the menstrual cycle. It receives information from other parts of the nervous system (e.g. olfactory and visual data), and receives information concerning the level of hormones in its perfusing blood. Although the exact anatomical site of the receptor cells in the hypothalamus which are responsive to hormone levels has not yet been established, it is clear that the hypothalamus is capable of integrating this information, and responds by liberating specific "releasing

factors" into the portal circulation surrounding the pituitary stalk. The importance of this portal circulation in the humoral communication between the hypothalamus and anterior pituitary was first demonstrated in animal experiments by Everett and Nikitovitch-Winer (1963).

The existence of specific hypothalamic releasing factors which cause the release of individual anterior pituitary hormones was first revealed by the isolation of Corticotrophin Releasing Factor (CRF). It has been shown that this octapeptide is a potent liberator of ACTH from the anterior pituitary. Subsequently, considerable study has been devoted to the isolation and identification of other hypothalamic releasing factors. To date, releasing factors have been identified for Adrenocorticotrophic Hormone (ACTH), Thyroid Stimulating Hormone (TSH), Growth Hormone (GH), Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH). A sixth factor concerned with the secretion of Luteotrophic Hormone (Prolactin) has been identified, and has been shown to be inhibitory, rather that facilitatory to the secretion of the anterior pituitary hormone.

The hypothalamic releasing factors are probably all polypeptides of low molecular weight (1,000-2,000 or less), and are not species specific. For example, Luteinising Hormone Releasing Factor (LH-RF) from pigs, sheep and cattle

has been highly purified, and it has been shown that the injection of this animal LH-RF into children, adult males, and adult females causes a rapid rise in the blood level of LH (McCann and Porter, 1969).

Factors Affecting the Secretion of LH-RF and FSH-RF

Since the secretion of LH and FSH from the anterior pituitary depends upon stimulation by specific hypothalamic releasing factors, it appears that there is some intrinsic mechanism within the hypothalamus, whereby the secretion of FSH-RF and LH-RF occurs in the correct sequence. This mechanism has been clearly named the "hypothalamic clock" by Harris (1969). The operation of this "clock" may be influenced by the level of ovarian hormones in the blood, and by stimuli received through the neural connexions of the hypothalamus with other parts of the nervous system. In the human female, humoral factors appear to be of greater importance than neural influences in the secretion of FSH and LH.

The precise nature of the feedback control of ovarian steroids on the hypothalamic-hypophyseal system is under investigation. It has been shown that the mid-cycle peak in LH is preceded by a rapid rise in plasma oestradiol, which reaches a peak 0-5 days before the L.H. peak (Corker, Naftolin and Exley, 1969; Dufau, Dulmanis, Hudson, Catt, Fullerton and Burger, 1970). These workers have suggested that the rapidly rising blood

level of oestradiol, produced by the secretion of the developing follicle, is the stimulus for pituitary LH release and the succeeding ovulation. This hypothesis is supported by the observation that administration of oestradiol stimulates LH release in sheep and in man. The release of LH from the pituitary may be effected through the action of a rapidly rising blood oestradiol concentration upon the hypothalamus, thereby provoking the secretion of LH-RF.

Variations in Plasma Levels of FSH, LH, Oestradiol and Progesterone During the Normal Menstrual Cycle

A. Pituitary Gonadotrophins

FSH

Radioimmunoassay studies have shown that in the early follicular phase, FSH levels are raised. There follows a gradual decline, until a pre-ovulatory nadir is reached; then, at the time of ovulation, FSH levels reach a peak which appears to accompany the LH peak. This pattern as described by Faiman and Ryan (1967), Franchimont (1966) and Cargille, Ross and Yoshimi (1969) appears to be a consistent one. The latter investigators also described suppression of FSH values during the luteal phase to levels significantly below those of the late follicular phase, and a late luteal rise before the onset of menstruation. The significance of the mid-cycle peak of FSH is unknown. Lostroh (1966) has shown

that in the rat, a surge of either FSH or LH can serve as the stimulus for ovulation, after follicular maturation has been induced by a combination of FSH and LH. Whether this may be true for man is unknown.

LH

The existence of a marked mid-cycle peak in LH secretion has been reported by several workers (e.g. Odell, Ross and Rayford, 1966; Goebelsmann, Midgley and Jaffe, 1969). It is presumed that this mid-cycle LH peak is the stimulus for ovulation. Following the ovulatory LH peak, both LH and FSH remain at lower levels during the luteal phase than during the follicular phase (Saxena, Demura, Gandy and Petersen, 1968).

B. Ovarian Hormones (Only the principal physiologically active hormones, oestradiol and progesterone, will be discussed here).

Oestradiol

Earlier urinary excretion studies (Brown, 1955) suggested that a rise in oestrogen secretion was associated with follicular development, and preceded the ovulatory LH peak. More recently, measurement of oestradiol in plasma, first by a double-isotope technique (Baird 1968), and later by competitive protein-binding radioassay (Korenman, Perrin and McCallum, 1969; Corker et al., 1969; and Dufau et al., 1970)

has revealed a consistent pattern. During the follicular phase, a gradual increase occurs, until the great rise, which appears up to 5 days before the ovulatory LH peak. Following the pre-ovulatory peak, oestradiol levels fall rapidly to reach early follicular phase levels. During the luteal phase, a secondary rise occurs, followed by a fall just prior to menstruation. The timing of the pre-ovulatory peak of oestradiol in relation to the ovulatory LH peak has been studied by Corker et al (1969) and Dufau et al (1970). The oestradiol peak has been found invariably to precede the ovulatory LH peak, usually by 24-48 hours. The role of this oestradiol peak in evoking LH secretion has been considered above.

The mean level of oestradiol in plasma is significantly greater during the luteal phase compared to the follicular phase. This is attributable to the secretory activity of the corpus luteum.

Progesterone

Daily estimations of plasma progesterone throughout the menstrual cycle have been reported by several workers (e.g. Neill et al., 1967; Yoshimi and Lipsett, 1968; and Johansson, 1969). During the first half of the cycle, levels remain low (1 nanogram/ml or less). There is a small rise during the few days before ovulation (Runnebaum and Zander, 1967),

which is believed to be caused by the secretion of progesterone by the follicular cells. Following the mid-cycle LH peak, progesterone levels rise rapidly, and remain high (above 5 ng/ml) until a rapid decline occurs in the premenstrual phase. At the onset of menstrual bleeding, progesterone values are those of the follicular phase in the majority of women (Johansson, 1969)

The mid-cycle peak in LH invariably precedes the rapid increase in plasma progesterone (Neill et al., 1967; Yoshimi and Lipsett, 1968); it appears unlikely, therefore, that a rising level of progesterone may stimulate LH or FSH release. On the contrary, it appears likely that pre-ovulatory progest--erone secretion may be stimulated by LH. The maintenance of high levels of progesterone during the luteal phase, at a time when both FSH and LH levels are low, suggests that continuous stimulation of the corpus luteum by LH is not necessary to its normal function.

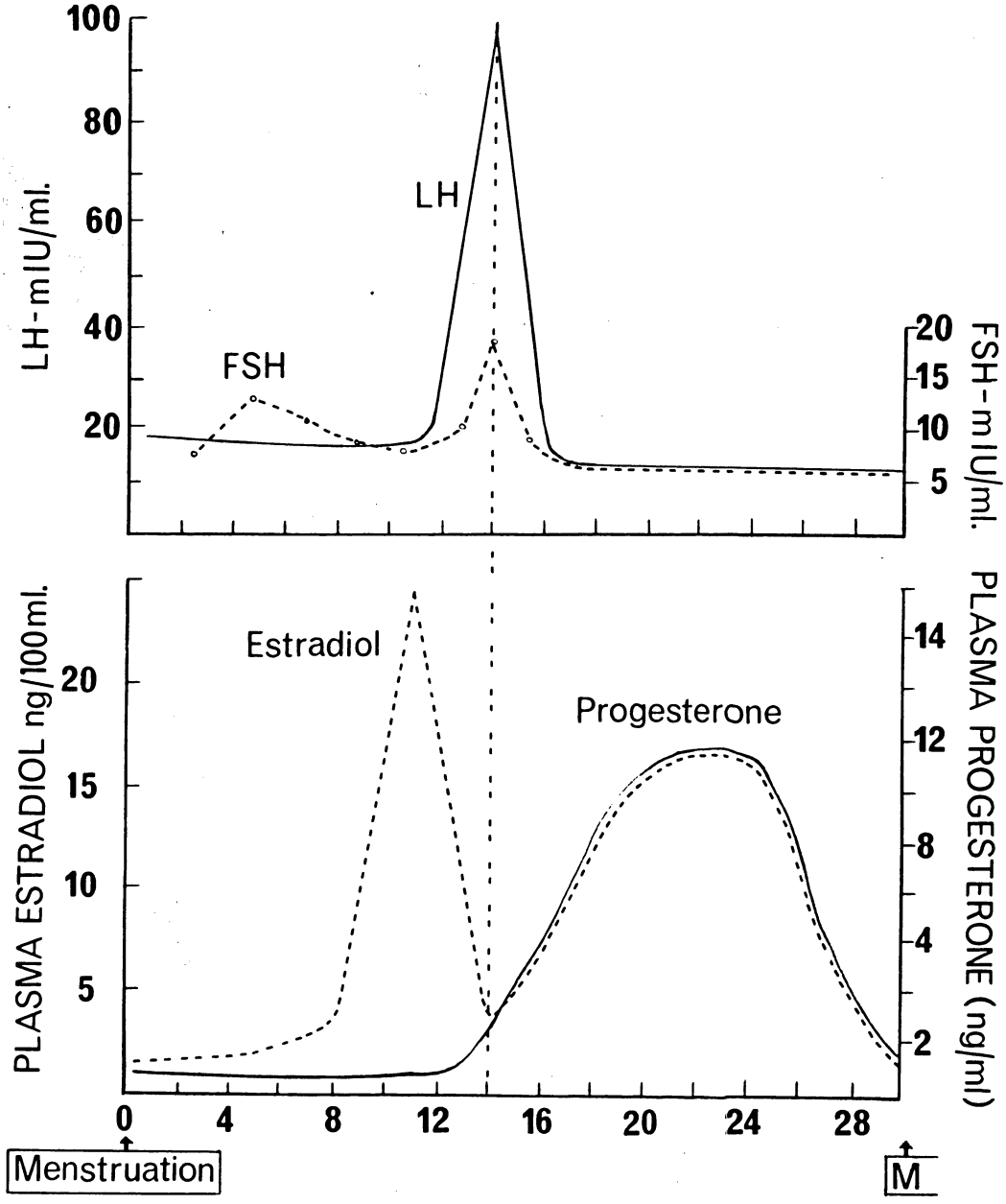


Figure 1.1 - Patterns of Circulating Gonadotrophins and Ovarian Hormones during the normal Menstrual Cycle

The Ovarian and Endometrial Cycles

Morphological changes are observed in the ovaries and uterine endometrium throughout the menstrual cycle. The ovarian changes may be described as the following phases: follicular phase, ovulation, luteal phase, and the phase of regression of the corpus luteum.

A. The Ovarian Cycle

Follicular Phase

The primordial follicle, which has already begun developing during the last phases of the preceding cycle, undergoes rapid growth under the influence of FSH. The enlarging ovum becomes surrounded by the zona pellucida, and is in turn surrounded by a layer of proliferating granulosa cells. As this phase progresses and the time of ovulation approaches, the follicle becomes distended with fluid secreted by the granulosa cells. The mesenchyme external to the follicle differentiates to form a thin vascular layer, the theca interna, which is an important source of oestrogen secretion.

Ovulation

The rapidly rising level of oestradiol in the blood, caused by the secretion of the theca interna, provokes a marked peak of LH secretion, together with a smaller peak of FSH secretion. It is believed that this mid-cycle LH peak is the

stimulus which provokes the rupture of the follicle and ovulation, although the mechanism whereby this is achieved is obscure. Following the release of the ovum from the ovary, the follicle becomes transformed into the corpus luteum. The granulosa cells become converted into large cells full of yellowish pigment, and the corpus luteum so formed becomes vascularised by the ingrowth of fine vessels from the theca interna.

The Luteal Phase

The corpus luteum reaches its maximum histological development 7 days after ovulation (Corner, 1956). It is at this time that maximum blood concentrations of progesterone are achieved in the majority of women (Johansson, 1969). The duration of secretory activity of the corpus luteum is a fairly constant 12-14 days. There is considerable evidence that the corpus luteum functions autonomously (Short, 1964). This view is further supported by observations that ovulation and pregnancy may be produced in hypophysectomised women by treatment with short courses of gonadotrophins (Bettendorf, 1963).

Regression of the Corpus Luteum

If fertilisation fails to take place, the corpus luteum degenerates rapidly in the few days preceding menstruation. This is accompanied by a rapid decline in plasma levels of both

progesterone and oestradiol. Korenman (1969) has suggested that this decline in oestradiol secretion may be the signal for increased pituitary gonadotrophin secretion, stimulating the development of the next follicle.

B. The Uterine Cycle

A detailed discussion of the endometrial changes which occur during the menstrual cycle is outside the scope of this thesis. However, the mechanism of menstrual bleeding deserves special consideration in view of the postulated relationship between the development of endometrial arterioles and migraine (Grant, 1965, 1968).

The Mechanism of Menstrual Bleeding

The endometrium is supplied by two sets of arteries, the straight arteries (which supply the basal layers), and the coiled arteries, which supply the superficial layers of the endometrium. Unlike the straight arteries, which undergo little change during menstruation, the coiled arteries undergo marked changes during the premenstrual and menstrual phases (Daron, 1936).

As the luteal phase progresses, these arteries become progressively more coiled, as their length increases disprop-ortionately to the thickening of the endometrium. This coiling is accentuated by regression of the superficial layers of the endometrium in the premenstrual phase. Markee (1940, 1946) has provided valuable experimental observations on the mechanism

of menstrual bleeding. He studied vascular changes in implants of endometrial tissue in the anterior chamber of the eye in Rhesus monkeys. The following description is based upon his findings.

As the coiled arteries increase in length, the blood flow through them slows. Vascular stasis is then increased by intense constriction of these arteries, beginning 4-48 hours before the onset of bleeding. As the vessels do not constrict synchronously, the subsequent bleeding and shedding of endometrium occurs first from one area, then from another. Markee observed 5 types of haemorrhage in the endometrial implants:

(a) Blood that escapes through a break in the wall of an arteriole or capillary may form a haematoma which ruptures. This is believed to be the most important mechanism.

(b) Blood may break through the uterine epithelium and escape without forming a haematoma.

(c) Diapedesis may occur through the wall of a capillary, and the escaping blood may or may not form a haematoma.

(d) There may be either a direct flow or reflux of blood from the veins in fields of previous haemorrhage and destruction of tissue

(e) Secondary haemorrhage may occur from the arterioles.

CHAPTER 2

OESTROGENS

2. OESTROGENS

Introduction

Prior to 1953, the only oestrogens known to be present in human urine were oestrone, oestradiol, and oestriol. Since then, many other steroid compounds possessing oestrogenic activity have been isolated. In 1964, the number of these compounds exceeded 20 (Breuer, 1964). However, the 3 "classical" oestrogens (oestrone, oestradiol and oestriol) are still considered the most important oestrogens in man. Of these, oestradiol has been shown to be the most potent by bioassay in animals, and is generally regarded as the true oestrogenic ovarian hormone in man (Loraine and Bell, 1966).

Sources of Oestrogens in the Body

The main sources of oestrogen production in man are the ovaries and placenta. Smaller amounts of these hormones are produced by the adrenal cortex and the testes. This topic has been reviewed in detail by Diczfalusy and Lauritzen (1961). In the ovary, oestrogens are secreted both by the theca interna and the stratum granulosum. They are secreted by the maturing follicle, and by the corpus luteum after ovulation. It is now generally believed that the principal ovarian oestrogens are oestradiol and oestrone (Rabinowitz, 1956; Zander, Brendle, von Munstermann, Diczfalusy, Martinsen, and Tillinger, 1959).

During pregnancy, the placenta is the main source of oestrogen production. There is evidence that, during early pregnancy, the placenta "takes over" from the corpus luteum, and that from the second or third month onward, the placenta is the major source of oestrogens (Diczfalusy and Lindqvist, 1956; Levitz, Condon and Dancis, 1955). This theory of the limited life span of the corpus luteum receives support from the observations of Yoshimi, Strott, Marshall and Lipsett (1969) and Johansson (1969), who found that the plasma progesterone reached its lowest level between the 5th and 9th weeks of gestation.

There is considerable indirect evidence to suggest that the adrenal cortex is capable of synthesising these hormones in man. For example, small amounts of oestrone, oestradiol and oestriol may be recovered from the urine of post-menopausal or ovariectomised women. Also, persons suffering from certain neoplasms of the adrenal cortex may excrete large quantities of these oestrogens in the urine.

Biosynthesis of Oestrogens

The use of isotopic labelling of steroids and steroid-precursor compounds has resulted in major advances in the understanding of the biosynthesis of these hormones. In the following short review, emphasis will be placed upon the

biosynthesis of oestrogens in the normal, intact woman.

There are 3 sources of oestrogens:

(a) From acetate (Rabinowitz 1956; Wotiz and Lemon, 1958; Ryan and Smith, 1961).

(b) From cholesterol and progesterone (Werbin, Plotz, Leroy and Davis, 1957; Davis & Plotz, 1958; Ryan and Smith, 1961).

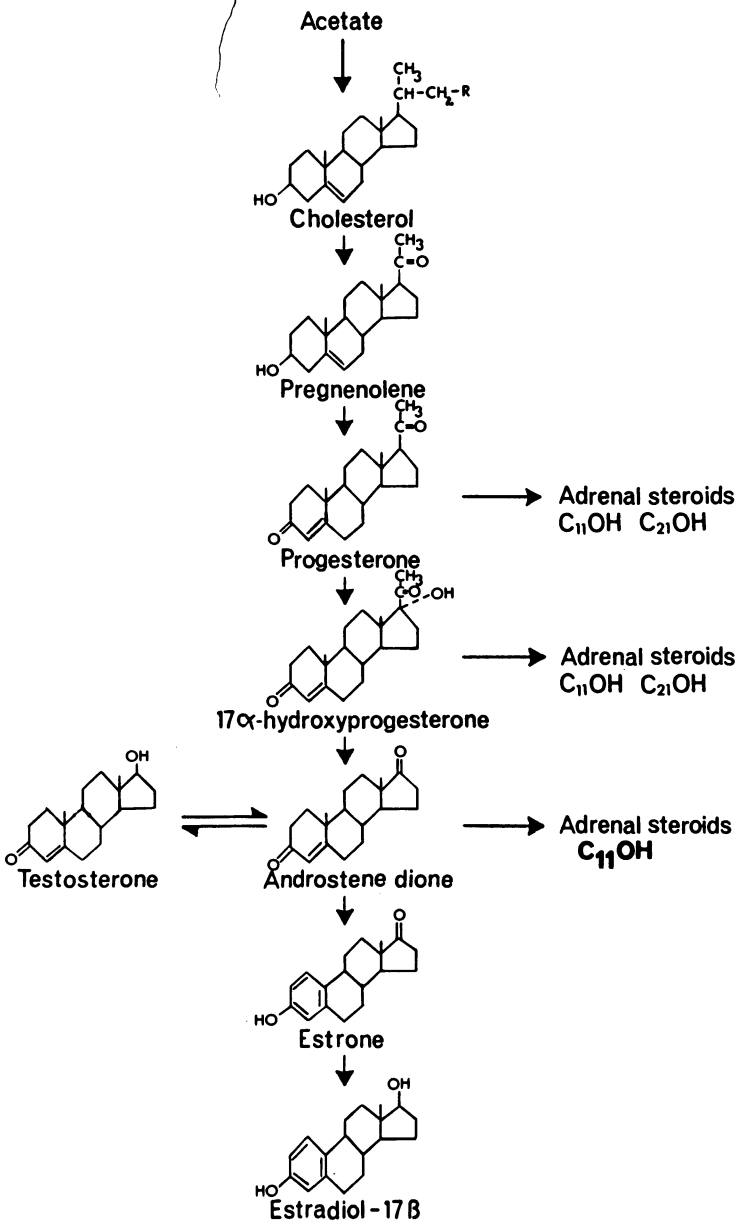
(c) By alteration of ring A of neutral C_{18} and C_{19} steroids from an aliphatic to an aromatic form. This process of "aromatisation" has been described by Meyer (1955), Ryan (1959) and Longchampt, Gual, Ehrenstein and Dorfman (1961).

The main pathway of oestrogen biosynthesis, according to Dorfman (1963) is:

ACETATE ⟶ CHOLESTEROL ⟶ ANDROGEN ⟶ OESTROGEN

BIOSYNTHESIS OF ESTROGENS

After SHORT, 1961



Conversion of Androgens to Oestrogens

The role of androgens as precursors in the synthesis of oestrogens was first suggested by Dorfman and Hamilton (1939). It has been confirmed subsequently by radioisotope studies in which testosterone was the substrate, and the products were oestrone and oestradiol. The conversion of androgens to oestro--gens was thus demonstrated in vitro by Baggett, Engel, Savard and Dorfman (1955). It is assumed that this reaction takes place mainly in the ovaries, but there is evidence that it may occur elsewhere in the body as well. Thus, after administering testosterone propionate to 2 adrenalectomised, ovariectomised women suffering from carcinoma of the breast, oestradiol and oestrone were isolated from the urine, whereas neither oestrogen had been detectable in the pre-treatment, control urine collections (West, Damast, Sarro and Pearson, 1956).

Metabolism of Oestrogens

The major site of oestrogen metabolism in the non-pregnant woman is the liver, although oestrogens may be metabolised by their target organs. Breuer, Knuppen and Haupt (1966), using a technique of incubating labelled oestrone and oestradiol with liver slices, have shown that these oestrogens are metabolised to a number of compounds, including those which have been described as the "newer" oestrogens (Breuer, 1964). Their work has raised the possibility that the "newer"

oestrogens may be metabolites of oestrone and oestradiol. They have also shown that the following metabolic steps involving oestradiol and oestrone may occur in the liver:

(a) Hydroxylation at positions 2, 6α, 7α, 15α, 16α and 16β.

(b) Methylation of the 2-hydroxyl group.

(c) Oxidation-reduction of the 17-hydroxyl and 17-oxo groups.

Oestrone and oestradiol are interconvertible (Ryan and Engel, 1953). Thus, oestrone and oestradiol form a reversible oxidation-reduction system, in which oestradiol is rapidly oxidised to oestrone, and more slowly reconverted to oestradiol (Gallagher, Fukushima, Noguchi, Fishman, Bedlow, Cassonto, Zumoff and Hellman, 1966). Apart from the liver and possibly the adrenal cortex, it has been shown that the interconversion of oestradiol and oestrone may take place in the uterine endometrium and myometrium during the proliferative phase (Sweat, Bryson and Young, 1967). These authors have shown that incubation of the uterine tissues with labelled oestradiol results in rapid conversion of oestradiol to oestrone. The capacity of the endometrium is about 40 times greater than that of the myometrium. The reverse reaction is of a much lower magnitude, indicating that the biotransformation is in favour of the formation of oestrone.

The subsequent metabolism of oestrone may occur along 2 pathways. The first of these, involving C_{16} hydroxylation, is quantitatively the most important (Brown, 1960), and results in the formation of oestriol. The second pathway, requiring C_2 hydroxylation, leads to the formation of 2-hydroxy oestrone, which in part is further metabolised by the addition of a methyl group at the C_2 oxygen function.

Conjugation of Oestrogens

Oestrogens are rendered water-soluble by conjugation (i.e., chemical combination with glucuronic acid or sulphuric acid) in order that they may be excreted in the urine. The 2 major forms of conjugation are:

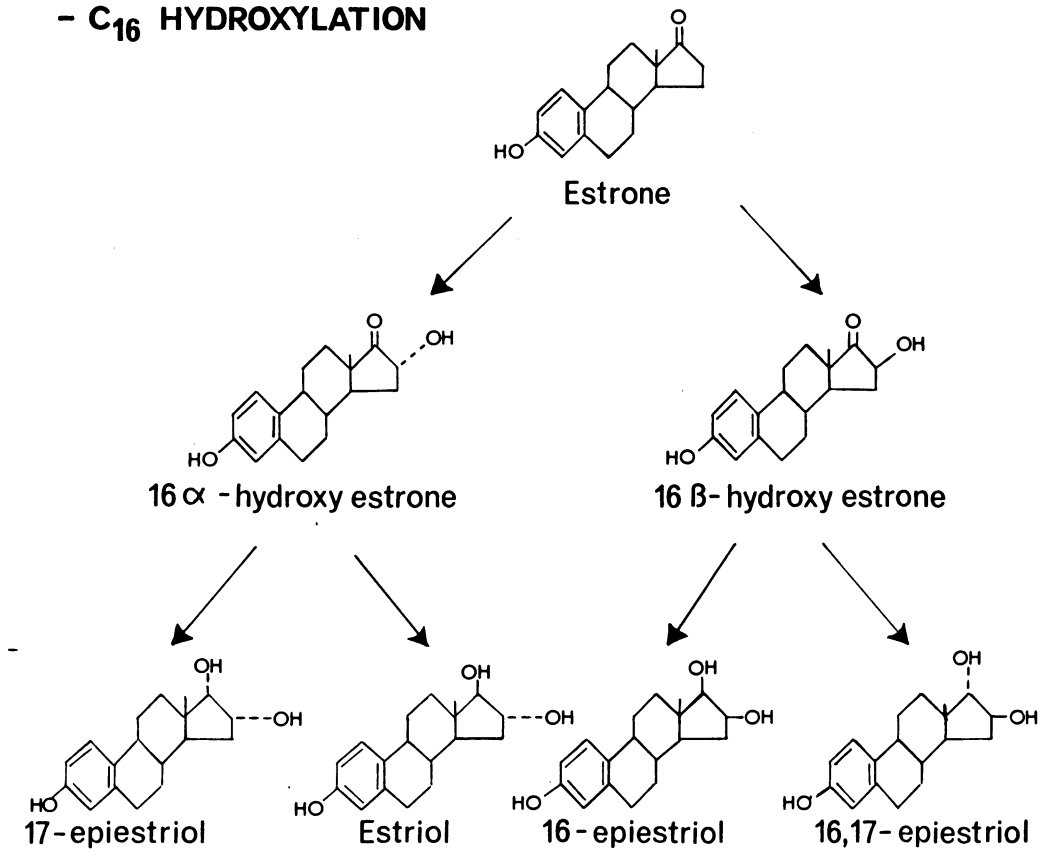
(a) Combination with glucuronic acid by an ether linkage analagous to the glucosides. The compounds formed are glucosiduronates.

(b) Esterification with sulphuric acid to form oestrogen hydrogen sulphates.

Conjugation is believed to be carried out mainly in the liver. The formation of oestrogen glucosiduronates by the liver has been demonstrated in vitro (Crepy, 1946) and in vivo in an isolated jejunal loop preparation (Diczfalusy and Lauritzen, 1961). The structural formulae of the 2 most important urinary oestrogen conjugates are shown.

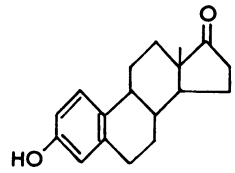
METABOLISM OF ESTRONE TO ESTRIOL (after Breuer, 1962)

- C_{16} HYDROXYLATION

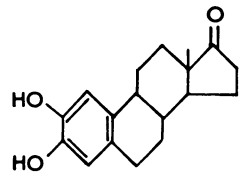


METABOLISM OF ESTRONE - C_2 HYDROXYLATION

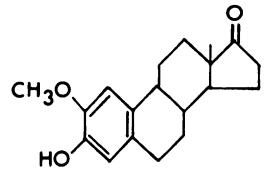
(After Gallagher et al, 1966)



Estrone

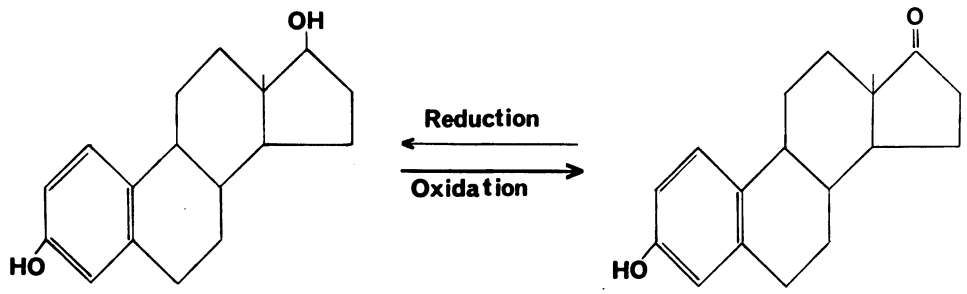


2-hydroxy estrone



2-methoxy estrone

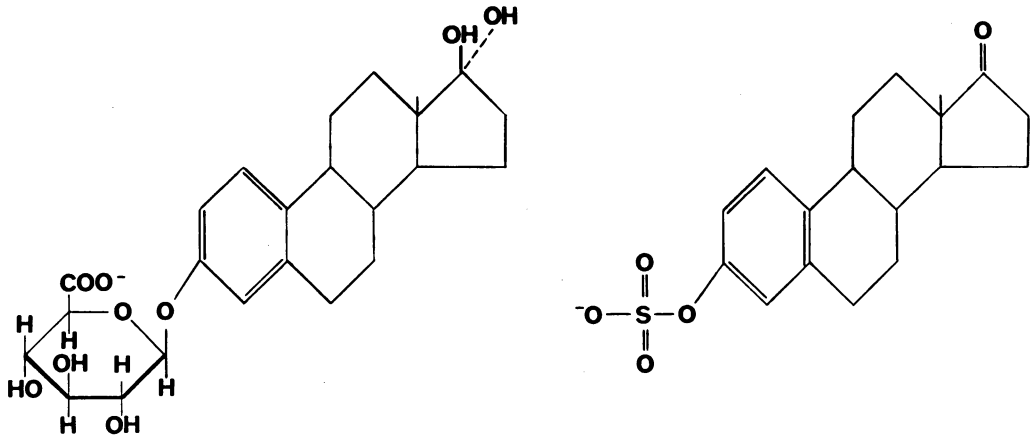
INTERCONVERSION OF OESTRADIOL AND OESTRONE



Oestradiol

Oestrone

URINARY OESTROGEN METABOLITES



Oestriol glucuronide

Oestrone sulphate

Transport of Oestrogens

The blood level of oestrogens represents an equilibrium between their rates of secretion by the endocrine glands, and their utilisation or removal by other organs. Electro-phoretic studies have demonstrated that oestradiol is bound by a beta globulin. The specificity of this protein has been studied by Murphy (1968), who has shown that it possesses a high degree of affinity for oestradiol and testosterone. In addition, both free and conjugated oestrogens are carried bound in loose combination to albumin (Slaunwhite and Sandberg, 1958). The significance and extent of protein-binding of oestrogens, and the association between oestrogens and red cells remains unclear. It is likely that the protein-bound hormone is in equilibrium with the free hormone, and thus constitutes a store from which free oestrogen could readily be mobilised.

The existence of an entero-hepatic circulation of oestrogen was first suggested by Cantarow, Rakoff, Paschkis, Hansen and Walkling (1943), and has been confirmed by radioisotope studies (Sandberg and Slaunwhite, 1957).

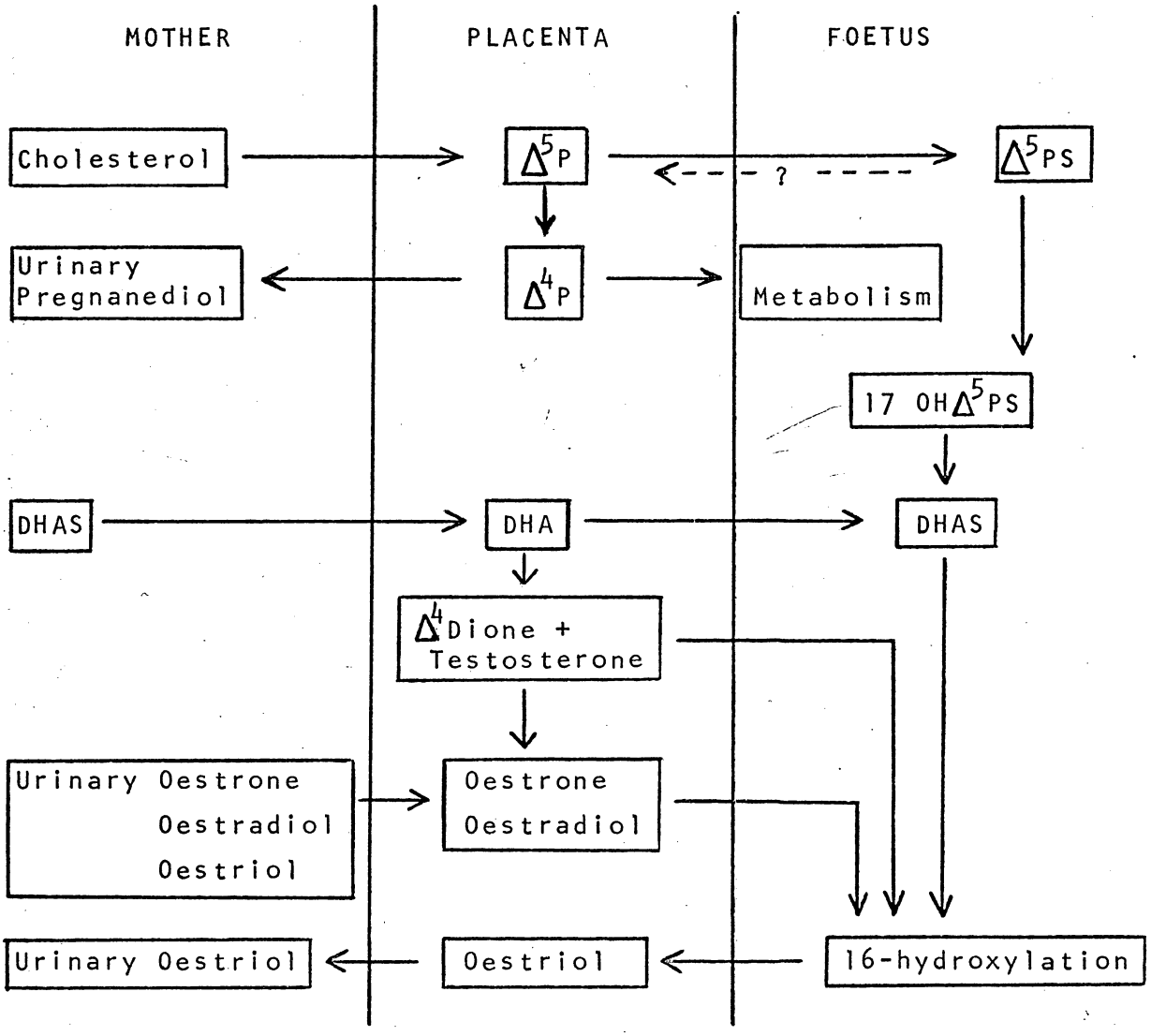
Oestrogen Metabolism During Pregnancy

Most of the studies of oestrogen metabolism during pregnancy have been concerned with the middle and third trimesters. Consequently, little is known of the mechanisms of oestrogen metabolism during early pregnancy. In middle and late pregnancy, the placenta is the principal source of the greatly increased production of oestrogens. The ability of the placenta to synthesise oestrogens from labelled androgens (Δ^4-androstenedione and testosterone) has been demonstrated in vitro by Ryan (1959). However, it appears that the placenta is not capable of synthesising oestrogens from acetate to any significant degree. It is likely that placental oestrogens are formed from maternal cholesterol, via preg--nenolone, progesterone, and the androgens. It has been suggested that another major substrate for placental oestrogen production may be dehydroisoandrosterone, derived from the maternal circulation (Solomon, 1966).

The foetal tissues are capable of conjugating oestrogenic steroids. This may serve as a protective mechanism, shielding the foetus from exposure to high levels of oestrogen. For example, the foetal liver conjugates oestrogens brought to it by the umbilical circulation, while the foetal lungs and gastrointestinal tract inactivate oestrogens present in the amniotic fluid (Levitz, 1966). The foetus inactivates these steroids mainly by the formation of sulphate conjugates.

Integrated Pathways for the Biosynthesis of Neutral Steroids and Oestrogens in the Foeto-Placental Unit at Mid-Pregnancy

(After Solomon, 1966)



KEY:

Δ^5 = Pregnenolone

Δ^4 = Progesterone

DHA = Dehydroisoandrosterone

DHAS = Dehydroisoandrosterone sulphate

Δ^4dione = Androstene dione

17 OH ^{5}PS = 17-hydroxyprogesterone sulphate

These conjugates are then cleaved, and transported back in the free form to the maternal circulation.

Blood Oestrogen Levels During Pregnancy

Recent studies of the blood levels of oestrogens during pregnancy (e.g., Roy, Harkness and Kerr, 1963) have confirmed the earlier conclusions based upon urinary excretion studies (e.g. Bradshaw and Jessop, 1953; Brown, 1956), which showed that there is an early and progressive rise in the secretion of oestrone, oestradiol and oestriol following fertilisation. Blood oestrogen estimations have shown that oestrone and oestradiol rise steadily through the first and second trimesters, with a generally lessened rise or "plateau" effect seen after about the 34th week. Blood levels of oestriol however, begin to rise much more rapidly after the 32nd week (Roy et al., 1965). Smith (1966) found that plasma concentrat-ions of oestradiol did not rise significantly during the final 6 weeks of pregnancy. Similarly, plasma oestradiol levels were measured serially in a number of women during late pregnancy and in labour by Rado, Crystle and Townsley (1970), who found that values generally remained "steady" during the last 2-3 weeks of pregnancy.

The Physiological Actions of Oestrogens

A detailed discussion of this topic is outside the scope of this thesis, but the physiological actions of the oestrogens will be considered briefly below. The subject has been reviewed by Lloyd (1966).

A. GENITAL EFFECTS

1. Endometrium: Oestrogens stimulate mitotic activity, causing thickening of the endometrium, with an increase in its content of water, electrolytes, protein, nucleotides, and a number of enzymes. This produces the typical "proliferative" histological appearance during the first half of the cycle.

2. Cervix: The cervical mucus becomes watery, and its secretion is increased under the influence of oestrogen.

3. Vagina: Oestrogens cause thickening of the vaginal mucosa. The vaginal epithelial content is increased, and the pH of the vaginal secretions is reduced through the increased production of lactic acid by the bacilli of Doderlein.

4. Myometrium: In addition to stimulating mitotic activity, oestrogens increase myometrial contractility.

5. Breasts: Oestrogens stimulate the development of the duct system of the breasts.

B. EXTRA-GENITAL EFFECTS

1. Anabolic action: Oestrogens have a general anabolic action, particularly on the female secondary sex organs. Diminution of oestrogen secretion post--menopausally may be responsible for the loss of protein matrix of bone, leading to osteoporosis.

2. Plasma proteins: Oestrogens increase the levels of thyroid-binding and corticosteroid-binding globulins.

3. Water retention: The retention of sodium and water is stimulated by oestrogens.

4. Action on Vascular Smooth Muscle: This topic is discussed later in this thesis (p. 146).

The many systemic changes which occur during the menstrual cycle, and which therefore appear to be caused by fluctuations in plasma levels of oestrogens and progesterone have been reviewed by Southam and Gonzaga (1965).

The other effects of oestrogens (such as their effect upon the coagulation mechanism) do not seem to fall within the scope of this work, so that a discussion of these effects has been omitted.

CHAPTER 3

PROGESTERONE

PROGESTERONE

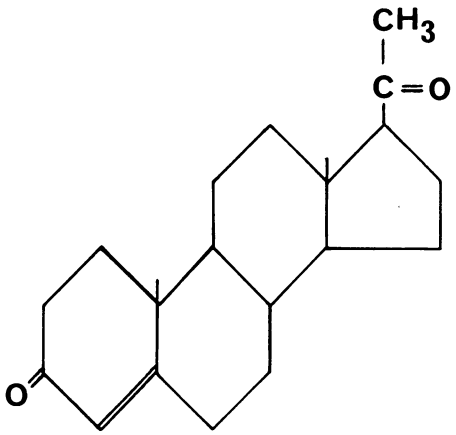
Introduction

Progesterone was first isolated from extracts of sows' corpora lutea almost simultaneously by 4 groups of workers independently (Butenandt, Westphal and Hohlweg, 1934; Slotta, Ruschig and Fels, 1934; Allen and Wintersteiner, 1934; and Hartmann and Wettstein, 1934). Its chief urinary metabolite, pregnanediol, had been isolated from human pregnancy urine several years earlier (Marrian, 1929). Shortly after progesterone had been isolated, its structure was completely elucidated by Butenandt and Schmidt (1934) and by Fernholz (1934)

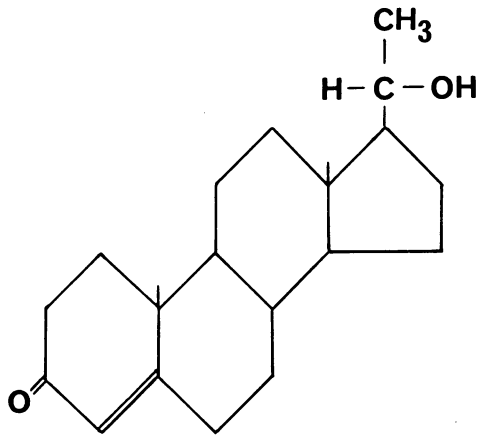
Sources of Progesterone in the Body

1. The Ovary: Measurement of progesterone in ovarian venous blood has shown that the ovary secretes most of the progesterone in the non-pregnant woman (Mikhail, Zander and Allen, 1963). Although it is generally agreed that the corpus luteum is largely responsible for this secretion of progesterone, there is evidence that the developing follicle may also be capable of secreting progesterone. Zander and von Munstermann (1956) isolated progesterone from follicular fluid, and showed that the concentration of progesterone within the follicle was high immediately before ovulation. The same workers found that

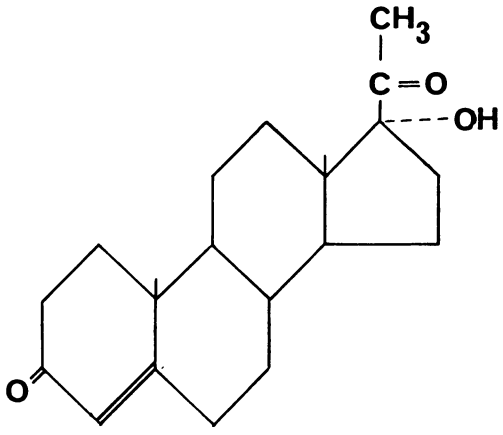
STRUCTURAL FORMULAE OF PROGESTERONE AND ITS CHIEF METABOLITES



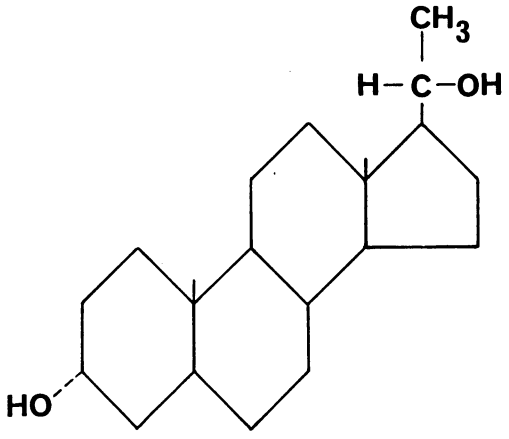
Progesterone



20α dihydroprogesterone



17α hydroxyprogesterone



Pregnanediol

the progesterone content of the corpus luteum paralleled its functional activity, being maximal on days 21 and 22 of the cycle. It is believed that the granulosa (lutein) cells of the corpus luteum are responsible for progesterone secretion, while the theca cells secrete oestrogens (Short, 1961).

2. The placenta: The placenta is the major source of progesterone from the 8-9th week onward. The pattern of progesterone in the blood during pregnancy will be described separately.

3. Adrenal cortex: The key role of progesterone in the biosynthesis of most of the adrenal cortical hormones has been established (Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker and Pincus, 1951). Progesterone has been isolated from adrenal venous blood (Short, 1960). Klopper, Strong and Cook (1957) have shown that the intravenous injection of ACTH causes a marked increase in the urinary excretion of pregnanediol. Since pregnanediol is the major metabolite of progesterone, this experiment illustrates that the secretion of progesterone by the adrenal cortex is stimulated by ACTH.

Although the adrenal cortex synthesises progesterone, it metabolises it further to produce most of its steroid hormones. The quantity of progesterone released into the

circulation from the adrenal cortex is, therefore, small, and makes only a minor contribution to the plasma level of progesterone. In normal women, plasma progesterone levels reflect almost entirely the secretion of the ovaries and placenta.

Biosynthesis of Progesterone

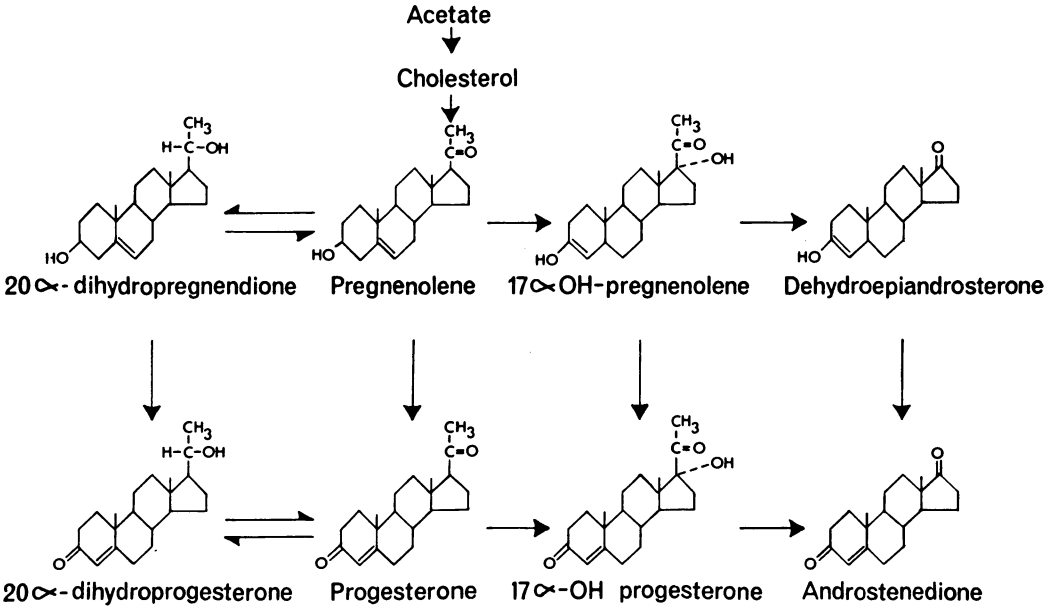
There is evidence that progesterone is synthesised by all the steroid-producing glands via the following pathway (Samuels, 1955):

ACETATE → CHOLESTEROL → PREGNENOLONE → PROGESTERONE

Progesterone probably acts as the key metabolic intermediate from which all adrenal corticoids, androgens and oestrogens are derived (Short, 1961).

Ryan and Petro (1966) devised a technique for separating the granulosa cells from the theca cells of the ovarian follicle. In this way, they were able to study the metabolism of labelled progesterone and pregnenolone by the granulosa cells and the theca cells separately. They found that the granulosa cells converted pregnenolone to progesterone, 17α-hydroxyprogesterone, and oestrone, and converted progesterone to 17α-hydroxyprogesterone and oestrone.

PATHWAYS FOR THE BIOSYNTHESIS OF OVARIAN STEROIDS FROM PREGNENOLENE AND PROGESTERONE (after MacAulay & Weliky, 1968)



Conversely, the thecal cells converted pregnenolone to progesterone, 17α-hydroxyprogesterone, androstenedione, and oestradiol, and progesterone was converted to the last 3 compounds. The conversion of pregnenolone to progesterone was much more rapid with the granulosa cells than with the thecal cells, while indications of an alternative pathway via Δ^5 compounds were much more apparent than with the thecal cells.

Metabolism of Progesterone

The role of progesterone in the synthesis of adrenal corticoids, androgens and oestrogens has been described. Progesterone which is not utilised in this manner is metabolised, mainly by the liver. There is some evidence that progesterone may be metabolised, to a limited extent, by extrahepatic tissues, eg. by the endometrium during the proliferative phase (Bryson et al., 1967). The metabolic inactivation of progesterone requires the processes of reduction and conjugation.

(a) Reduction: Numerous reduction products of progesterone have been isolated from the urine of women, both pregnant and non-pregnant. The most important quantitatively are pregnanediol and 3αhydroxy-5βpregnan-20-one. The major reduction processes in the liver consist of reduction of the 20-ketone group to a 20-hydroxyl group, and reduction of the A ring of the steroid nucleus to various pregnane derivatives.

(b) Conjugation: The reduced progesterone metabolites are conjugated by the liver and excreted in the bile. As in the case of the oestrogens, there is a considerable entero-hepatic circulation of these metabolites. Pregnanediol is excreted in the urine conjugated with glucuronic acid at the 3 position as a glucuronide (Venning and Browne, 1936).

Fotherby (1964) has shown that there are 4 main pathways for the excretion of progesterone metabolites. The most important is via the urine, but these metabolites are also excreted in the bile and faeces, in the expired air, and from the skin.

Transport in the Blood

Progesterone is carried almost exclusively in the plasma, since there is very little combination with the red cells, even when they are incubated together for prolonged periods in vitro (Short, 1958), Progesterone is bound to plasma albumin (Westphal and Ashley, 1959), and also to corticosteroid-binding globulin (Sandberg, Rosenthal, Schneider and Slaunwhite, 1966). A third binding protein, an alpha$_1$ glycoprotein, binds both testosterone and progesterone (Westphal and Ganguly, 1968), but is probably of little significance under physiological conditions.

Plasma Progesterone During Pregnancy

Following fertilisation, there is an early rise in plasma progesterone to above luteal phase levels (i.e., above 20 ng/ml) This may be evident as early as 10 days after the mid-cycle peak in urinary oestrogen excretion (Johansson, 1969). Parallel rises in plasma levels of 17α-hydroxyprogesterone and chorionic gonadotrophin (HCG) accompany the rise in progesterone (Yoshimi et al., 1969). This increased secretion of HCG suggests that some of the early secretion of progesterone may be derived from the trophoblast of the embryo, as well as from the corpus luteum.

There is considerable evidence that the corpus luteum plays the major role in the secretion of progesterone up to about the 10th week of gestation. This hypothesis was originally suggested by studies of the effect on pregnancy of removal of the corpus luteum at various stages early in pregnancy (e.g., Froewis, 1963), and by histological studies of the corpus luteum of pregnancy (Gillman and Stein, 1941). It has been supported by more recent work involving the assay of progesterone in blood taken from the uterine and ovarian veins during pregnancy (Mikhail and Allen, 1967). After the initial rise in plasma progesterone, the concentration shows a gradual decline between the 5th and 9th weeks, until a nadir is reached (Yoshimi et al., 1969; Johansson, 1969). This decline

is believed to occur during the period of "take over", when the secretion of progesterone is transferred from the degenerating corpus luteum to the placenta.

A second, sustained surge in progesterone then progresses from this point onward. Plasma progesterone levels rise steadily, until by the 34th week, a "plateau" has been reached. Beyond this time, no further significant increase in plasma progesterone is observed (Johansson, 1969).

The Physiological Actions of Progesterone

This topic has been reviewed concisely by Lloyd (1966). The known actions of progesterone under physiological conditions may be enumerated as follows:

A. GENITAL EFFECTS

1. Endometrium: After priming with oestrogen, progesterone promotes endometrial thickening, with lengthening and dilatation of the spiral arterioles, and dilatation of the endometrial glands, resulting in the "secretory" histological pattern characteristic of the luteal phase.

2. Myometrium: Progesterone affects myometrial contractility, depressing the response to oestrogens, possibly by altering the membrane potential of the myometrial cells.

3. Breasts: Progesterone stimulates development of the lobulo-alveolar system of the breast.

4. Maintenance of Pregnancy

B. EXTRA-GENITAL EFFECTS

1. Catabolic action: Progesterone accelerates the catabolism of protein. Its administration causes an increase in the urinary excretion of nitrogen, and a lowering of the plasma concentration of several amino acids (Landau and Lugibihl, 1967).

2. Antagonism of Aldosterone: Progesterone antagonises the effect of aldosterone on the renal tubules, and therefore promotes the renal excretion of sodium (Landau and Lugibihl, 1961).

3. Effects of Progesterone on vascular smooth muscle

This topic will be considered later in this thesis (p. 146).

CHAPTER 4

THE MEASUREMENT OF PROGESTERONE AND OESTRADIOL IN BLOOD

A. THE MEASUREMENT OF PROGESTERONE IN BLOOD

Introduction

The measurement of progesterone in plasma by chemical methods began with the work of Zander and Simmer (1954), who described a method based on ultraviolet spectroscopy. Up to this time, estimates of progesterone secretion depended upon the measurement of urinary excretion of pregnanediol, the chief metabolite of progesterone. These urinary excretion studies were inferior to the measurement of the hormone in plasma, since they were subject to variations in hepatic metabolism and excretion by the kidneys; furthermore, there was an inevit--able latent period between a change in the blood level of progesterone, and its reflection as an increase or decrease in the urinary excretion of pregnanediol.

Although the early fluorimetric and colourimetric methods for measuring progesterone in plasma represented a significant advance over urinary pregnanediol excretion studies, they still suffered from important disadvantages. The most important of these were lack of specificity, and relative insensitivity. For example, in the original method of Zander and Simmer (1954), the lower limit of sensitivity was 0.05 micrograms of progesterone. Thus, a luteal phase level of 10 nanograms/ml would be detectable in a plasma sample of 5 ml; but lower levels of progesterone would require larger samples of plasma

for their measurement. Under these circumstances, daily blood sampling would have to be restricted to the luteal phase, and, because of the larger volumes of blood required, little information could be gathered about plasma progesterone levels during the follicular, peri-ovulatory, or premenstrual phases of the cycle.

Other fluorimetric techniques were described by Short (1958), Sommerville, Pickett, Collins and Denyer (1963), Heap (1964), and Touchstone and Murawec (1960). These techniques offered minor improvements in specificity and precision.

Gas-liquid chromatography was used to measure progesterone in plasma by Kumar, Ward and Barnes (1964). This resulted in a considerable improvement in sensitivity, which was further increased by the addition of electron-capture (van der Molen and Groen, 1965). The main disadvantages of gas-liquid chromatography were the laborious nature of the preliminary procedures, and the introduction of errors by contamination of the blood sample with sebum (Lurie, Villee and Reid, 1966).

Double-isotope methods for measuring progesterone in plasma were described by Woolever and Goldfien (1963), and Riondel, Tait, Tait, Gut and Little (1965). Although these methods were satisfactory from the viewpoints of specificity and accuracy, their laborious nature made them unsatisfactory for the

processing of large numbers of samples.

In 1963, Murphy, Engleberg and Pattee described a method for measuring hormones (including corticosteroids and progesterone) in plasma, based upon the principle of competitive protein-binding. This principle was first used to measure serum insulin levels by Berson and Yalow in 1957. Subsequently, competitive protein-binding assays have been devised which possess the required accuracy, specificity, and reproducibility to allow the accurate daily measurement of plasma progesterone throughout the entire menstrual cycle (e.g. Yoshimi and Lipsett, 1968). These techniques are particularly suitable to daily estimations, since they require very small volumes of plasma (0.5-1.0 ml) for each determination.

The Principle of Competitive Protein-Binding

The mechanism and kinetics of competitive protein-binding hormone assays have been considered at length by Murphy (1964), and more recently at the Second Karolinska Symposium on Reproductive Endocrinology (1970). The measurement of progesterone by this technique is based on the fact that cortisol-binding globulin (CBG) reversibly binds both progesterone and cortisol (or corticosterone, or 11-deoxy cortisol, which differ in structure from cortisol only by the position of a single hydroxyl group). Thus, progesterone and

cortisol and related corticosteroids compete for binding sites on the carrier CBG molecule.

If all the binding sites on the CBG molecule are occupied by an isotopically-labelled corticoid (e.g. corticosterone), and progesterone is then added to the solution in increasing amounts, an increasing number of labelled corticosterone molecules will be displaced from the carrier CBG molecule by the unlabelled progesterone. By devising a system for separating the displaced labelled corticosterone from the corticosterone still bound by CBG, it is possible to correlate the degree of displacement with the amount of progesterone present, thereby allowing an accurate measurement of progesterone to be made. In the method to be described, free labelled corticosterone was separated from CBG-bound corticosterone by gel filtration carried out using small columns of Sephadex.

Assay of Plasma Progesterone by Competitive Protein-Binding

The method described below was used throughout these studies, and is based upon the method described by Bassett and Hinks (1969) for the measurement of plasma corticoids. The thin-layer chromatographic step, where employed, was identical to that described by Neill et al. (1967).

Materials

Borate buffer, 0.05M, pH 7.6, was prepared from a stock solution of borate dissolved in double distilled water to 0.5M concentration, with pH adjusted to 7.6 by the dropwise addition of 5N NaOH. The buffer solution was kept refrigerated at all times, to avoid the risk of bacterial contamination.

Petroleum ether (Petroleum spirit), B.P. 40°-60°C

Ethanol, A.R., redistilled, was used in the preparation of progesterone standards.

Progesterone, crystalline, used in the preparation of standards, was obtained from Ikapharm (Israel) Ltd.

Sephadex, G-25 (fine) and G-25 (coarse) was obtained from Pharmacia (Uppsala)

All solvents were of analytical grade.

Isotopically-labelled steroids:

(a) Corticosterone 1,2-^{3}H, Specific Activity greater than 20,000 mCi/mM.

(b) Progesterone 7α-^{3}H, Specific Activity greater than 2,000 mCi/mM.

All radiochemicals were obtained from The Radiochemical Centre, Amersham, and were diluted with absolute ethanol and kept at 4^{o}C.

Thin layer chromatography (TLC) was performed on 20 x 20 cm Eastman "Chromogram" pre-coated silica gel sheets containing fluorescent indicator (Type K 301 R), washed in absolute methanol, and activated before use by heating to 80^{o}C in an oven for 1 hour.

Scintillation fluid: Toluene solution containing 1,4 diphenylbenzene 0.3% (w/v) and 1,4-bis-2-(5-phenyl--oxazolyl)-benzene 0.01% (w/v).

Preparation of CBG-^{3}H-corticosterone

Plasma was obtained by exsanguinating a heparinised, anaesthetised dog. After separation, the dog plasma was stored in 10 ml quantities at -10^{o}C, and thawed once only before use.

To achieve adequate sensitivity, the 10 ml of dog plasma were passed through a long column of Sephadex to remove most of the corticosteroids in it before use. This long column of Sephadex consisted of a glass tube, 1.75 x 85 cm high, packed with Sephadex G-25 (coarse), and surrounded by a water jacket maintained at 45°C. The dog plasma was washed into the column with a small amount of borate buffer, and eluted with the same buffer. The first 80-100 ml eluted were discarded ("void volume"). The protein-carrying fraction (approximately 40-50 ml) was collected and diluted to 500 ml with borate buffer.

In order to saturate the binding sites on the CBG molecules contained in this solution, 40 microcuries of 1,2-^{3}H corticosterone in 2 ml ethanol were deposited in a glass measuring cylinder, and the ethanol solvent was evaporated off under a stream of nitrogen. The diluted canine plasma was then added carefully to this cylinder, and gently rotated. The diluted plasma solution then contained approximately 80 nanocuries/ml, and was stored at 4°C. It was found to remain stable for several months.

Methods

Collection of Plasma Samples: Blood was obtained by venepuncture using a heparinised syringe, and plasma was

separated by centrifugation for 5 minutes at 2,000 r.p.m. To minimise any fluctuation in progesterone levels due to diurnal variation, venepunctures were performed between 8 and 9 a.m. Plasma obtained in this was was stored at -10°C until assay.

Extraction of Human Plasma: Samples of heparinised plasma, 0.5 to 1.0 ml (depending upon the expected concentration of progesterone) were extracted by shaking with 4 ml of petrol ether for 2 minutes, in tapered test tubes fitted with ground glass stoppers. For routine purposes, blanks were provided by extracting an equal volume of double-distilled water, and subjecting the extracts to the same procedures as the extracts from the plasma samples.

Plasma (aqueous) and petrol ether layers were separated by centrifuging for 5 minutes at 2,000 r.p.m. The top petrol ether layer was then transferred carefully by Pasteur pipette to round bottomed glass test tubes, also fitted with ground glass stoppers. Particular care was taken to avoid transferring any of the plasma layer.

Purification: The petrol ether extracts were washed with 2 ml double-distilled water, by shaking in the stoppered test tubes for 2 minutes. After removal of the bottom (aqueous)

phase, the procedure was repeated with a further 2 ml of distilled water. The tubes were then centrifuged to obtain a clear separation of aqueous and petrol ether phases. The petrol ether layer was then transferred as completely as possible to clean, round-bottomed test tubes. The extracts, together with the standards, were evaporated to dryness under a stream of nitrogen in a water bath maintained at 45°C.

Preparation of Standards: Crystalline progesterone was dissolved in redistilled A.R. ethanol, and diluted serially so that 0.2 ml of the standard solutions contained the following amounts of progesterone:

0 (i.e., 2 ml ethanol only), 0.125 ng, 0.25 ng, 0.5 ng, 1.0 ng, 2.0 ng, 4.0 ng, 8.0 ng, 12.0 ng, 16.0 ng, 24.0 ng, and 32.0 ng.

These standards were kept refrigerated at 4°C until use, and fresh standards were prepared every 3 months.

Using a glass micropipette, duplicate aliquots of 0.2 ml of each of the above standard solutions were added to round bottomed test tubes, and evaporated to dryness as described above. Because of the importance of the ethanol blank ("0" progesterone) as the reference point for 100%

binding of 1,2-^{3}H corticosterone to CBG, quadruplicate determinations of this standard were routinely performed.

Addition of 1,2-^{3}H corticosterone-CBG solution: To each tube containing the dried petrol ether extract of plasma, or the dried progesterone standard, 0.5 ml of the tritiated CBG solution (40 nanocuries) was added by glass micropipette. The contents of each tube were then mixed mechanically on a vortex mixer, and the tubes were transferred to a water bath maintained at 45^{o}C, to incubate for 15 minutes. At the end of incubation, the tubes were then mixed mechanically to ensure equilibration between the displaced and bound tritiated corticosterone. The tubes were then cooled for 20 minutes in iced water before transfer to the small Sephadex columns.

Separation of free from protein-bound ^{3}H-corticosterone

This was achieved by using a battery of 60 small columns, made from plastic syringes, with glass reservoirs attached, as described by Bassett and Hinks (1969). Each syringe was packed with 0.5G of Sephadex G-25 (fine), and maintained in a moist condition by periodic irrigation with cold borate buffer solution. In order to minimise the dissociation of labelled corticosterone from the CBG, these columns were kept in a walk-in cold laboratory. This stage of the assay required working at a room temperature of 4^{o}C.

The contents of each tube were transferred by Pasteur pipette to the top of individual small Sephadex columns, and the displaced borate buffer was allowed to run to waste. Each column was then eluted with 1.5 ml of cold borate buffer, and this eluate was collected in a glass counting vial.

Preparation for Radiation Counting: To each vial containing eluate, 8 ml of toluene scintillation fluid was added. The vials were then capped, and shaken vigorously for 1 minute to extract the isotope into the toluene phase. After cooling to $4^{\circ}C$ for at least 30 minutes, liquid scintillation counting was performed in a Packard TriCarb counter. Duplicate 10 minute counts were obtained for each sample.

After use, the small Sephadex columns were repeatedly washed with borate buffer, and could be re-used many times.

Preparation of Standard Curve: As the Sephadex columns retained the small, displaced molecules of labelled corticosterone, and allowed the large CBG molecules to pass through in the eluate, the radiation counts reflected the amount of ^{3}H-corticosterone still remaining bound to CBG. Thus, the greater the amount of progesterone present, the more displacement of labelled corticosterone from CBG would occur, resulting in lower counts of radiation obtained from

the column eluate.

To construct the standard curve, the percentage of binding of ^{3}H-corticosterone to CBG was plotted vs. progesterone concentration. The former value was obtained by calculating the ratio of:

$$\frac{\text{Counts from standard}}{\text{Counts from ethanol blanks}} \times \frac{100}{1}$$

These values, expressed as "percentage binding" of labelled corticosterone to CBG were plotted along the ordinate, while the progesterone concentration was plotted along the abscissa.

It can be seen from the accompanying table and graph that the addition of progesterone up to 8 ng produced a marked displacement of labelled corticosterone from its combination with CBG; however, after this point, the steep fall became flattened, so that relatively little further displacement occurred even when quite large further increments of progesterone were added. For this reason, suitable dilutions of plasma were prepared, according to the anticipated progesterone concentration, in order that the reading might fall on the steep (sensitive) part of the curve.

Thus, samples of plasma taken during the follicular phase were used undiluted, whereas samples obtained during the luteal phase were diluted 1:2 with double-distilled water. To assay plasma progesterone during late pregnancy, much greater dilution was required (up to 1:20).

Each assay included at least 2 distilled water blanks, and a control plasma. Because the displacement curve proved to be almost exactly reproducible from one assay to the next, the standard curve was not determined in all assays, but was determined at frequent intervals. There was, however, considerable variation in the dissociation curves for CBG when individual dog plasmas were compared; accordingly, when a new batch of CBG-^{3}H corticosterone was prepared, the standard curve required careful re-determination.

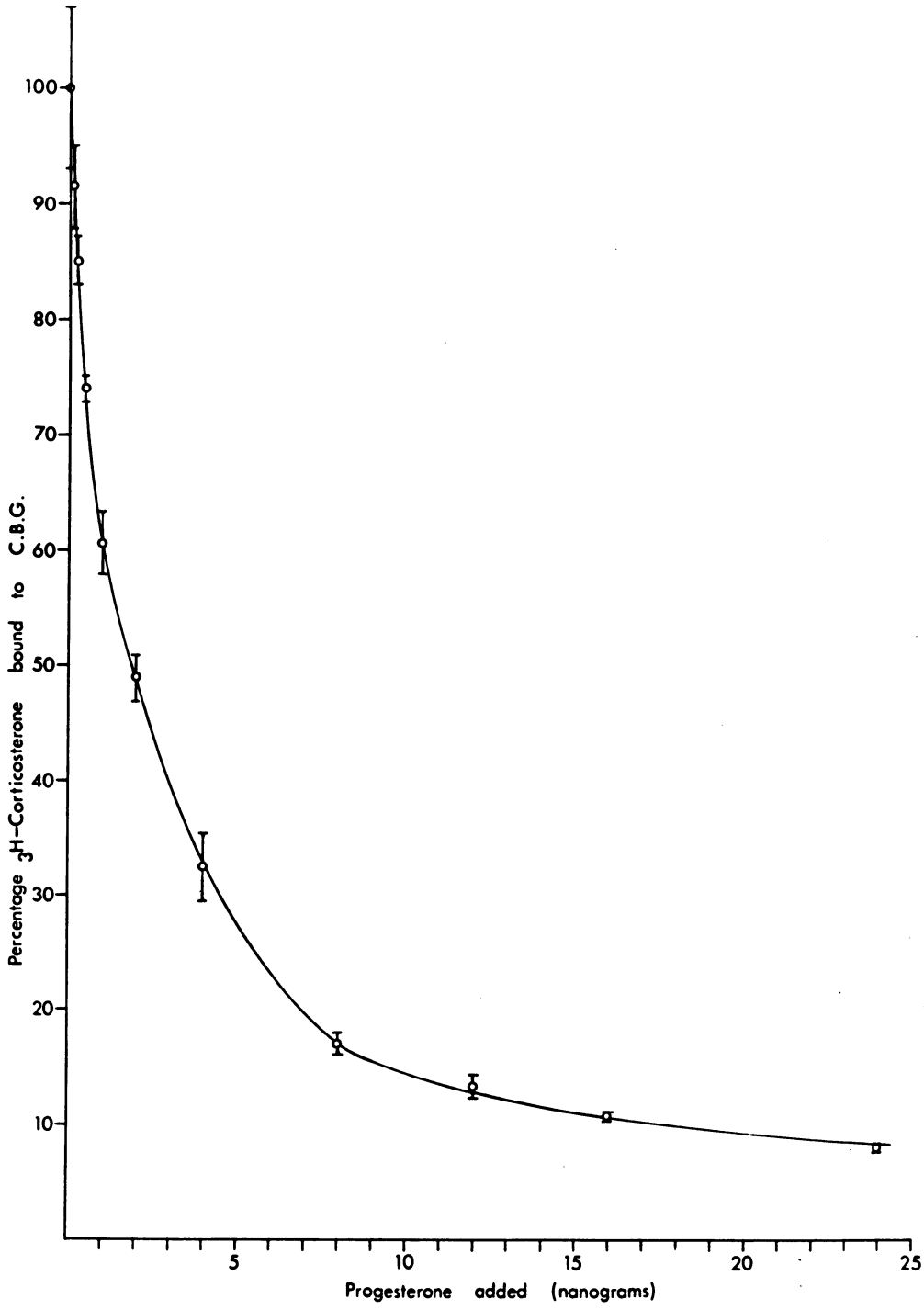
The blank value was deducted from each result. No correct-ion was made for procedural losses, except in the cases where the additional step of thin-layer chromatography (TLC) was required (such as with pregnancy plasma). Both of these aspects will be considered in detail below.

A typical standard curve is shown in the accompanying figure. Each point represents the mean of quadruplicate determinations, and the vertical bars indicate $\pm$ 1 standard deviation from the mean.

Table 4.1 - Data from which the standard curve for displacement of ^{3}H-corticosterone from CBG has been constructed

Progesterone standard (ng)	Counts per minute	Number of determinations	Mean % bound (± S.D.)
0	7,415 8,186 9,130 9,057 8,791 8,959	6	100 (7.3)
0.125	8,157 7,939 7,774 7,483	4	91.7 (3.8)
0.25	7,090 7,516 7,434 7,153	4	85.1 (2.5)
0.5	6,421 6,354 6,376 6,172	4	74.0 (1.2)
1.0	5,392 5,353 4,903 5,096	4	60.6 (2.7)

Progesterone standard (ng)	Counts per minute	Number of determinations	Mean % bound (± S.D.)
2.0	4,295 4,037 4,120 4,420	4	49.0 (2.0)
4.0	2,996 2,513 2,559 3,119	4	32.7 (3.6)
8.0	1,563 1,403 1,469 1,385	4	17.0 (0.9)
12.0	1,213 1,046 1,089 1,215	4	13.3 (1.0)
16.0	872 854 933 979	4	10.6 (0.7)
24.0	719 789 777 812	4	9.0 (0.4)


100
90
80
70
60
50
40
30
20
10
Percentage ^{3}H–Corticosterone bound to C.B.G.
5
10
15
20
25
Progesterone added (nanograms)

Evaluation of the Method

The method of assay for plasma progesterone as described was evaluated for accuracy, reliability, and specificity. Evaluation was considered necessary in view of the relatively recent introduction of competitive protein-binding forms of hormone assay, and to ensure that the thin-layer chromatography (TLC) step could be omitted from the assay of non-pregnancy plasma without loss of specificity or accuracy.

(a) Accuracy

The lower limit of sensitivity of the assay was set by the blank. In the routine assays in which distilled water was used as the blank, values for the blank ranged from 0 to 0.5 ng progesterone. Plasma obtained from an elderly, hypophysectomised male gave values indistinguishable from the blank. Because of this non-negligible contribution from the blank, it was considered desirable to determine the blank value with each assay.

When known amounts of progesterone were added to distilled water and assayed in this system, the following results were obtained.

TABLE 4.2

Progesterone added (ng)	Values obtained by assay (ng)	Mean	S.D.
2.0	2.7 2.5 2.1 2.2 1.5	2.2	0.48
4.0	3.7 3.6 3.9 3.2 4.1 4.1	3.8	0.35

The coefficient of variation of the estimation of 2.0 ng progesterone in this assay was 21.3%, and that of the estimation of 4.0 ng progesterone was 9.2%. It is evident therefore that there is greater variation between individual values at the lower progesterone levels. If 25% is taken as the maximum permitted coefficient of variation (as suggested by Baird, 1968), it would appear that in this assay system, the lower limit of sensitivity lies in the range 1.0-2.0 ng progesterone. For this reason, plasma progesterone values of and below 1.0 ng have been shown at the baseline in the following graphs.

(b) Reliability

The variation between values obtained for duplicate samples of plasma at different progesterone concentrations was studied. Both plasma samples of each duplicate pair were assayed in the same batch. In the following table, duplicate samples have been used to calculate standard deviations according to the formula:

$$S = \sqrt{\frac{d^2}{2n}}$$

where S = Standard deviation,

d = difference between duplicates

n = number of pairs of observations

(Braunsberg and James, 1961)

Table #3 shows the variation between duplicates when progesterone was determined in duplicate samples of plasma processed in the same assay.

Using a similar assay system to measure plasma cortisol in sheep, Bassett and Hinks (1969) found that the variation between duplicates processed in different batches was not greater than the variation between duplicates processed in the same batch. Accordingly, the difference between duplicates assayed in different batches was not investigated here.

TABLE 4.3 - PRECISION OF PROGESTERONE ASSAY

Range of plasma progesterone concentrations (ng/ml)	Duplicates within a single assay			
	Number of duplicate pairs	Mean concn. (ng/ml)	S.D.	Coefficient of variation
0-	13	1.57	0.34	21.7%
2-	10	4.14	0.42	10.1%
5-	15	7.78	0.75	9.7%
10-	11	13.0	1.22	9.4%
15-20	6	18.3	1.09	6.0%

(c) Specificity

The specificity of the assay for progesterone depends in part on the fact that only a limited number of steroids compete with 1,2-^{3}H corticosterone for binding sites on the CBG molecule (Murphy, 1967). Since cortisol and corticosterone are more effective competitors than proges--terone, they must be removed from plasma, leaving only progesterone to compete for binding sites. This is achieved by the preliminary extraction with petrol ether, which removes less than 0.2% of adrenal corticosteroids from plasma, while extracting 80-90% of the progesterone (Johansson, 1969). Any traces of cortisol or corticosterone are removed by the two washings with distilled water.

Petrol ether does, however, extract significant quantities of 17α hydroxyprogesterone and 20α dihydroprog--esterone. Both of these steroids are secreted by the corpus luteum (Zander et al., 1958; Mikhail et al., 1963), and both are capable of displacing labelled corticosterone from CBG (Johansson et al., 1968).

The displacement of labelled corticosterone from CBG by progesterone, 17α hydroxyprogesterone, and 20α dihydroprogesterone has been studied by the latter workers. As the displacement curve for 20α dihydroproges--terone is much flatter than that of progesterone, it does not

interfere seriously with the determination of progesterone by competition for binding sites. This has been confirmed by Johansson (1969). However, significant displacement of labelled corticosterone from CBG is caused by 17α hydroxy-progesterone. The plasma concentration of this steroid has been studied throughout the menstrual cycle by Strott and Lipsett (1968), who found a mean follicular phase level of 0.42 ± 0.2 ng/ml (n = 24), and a mean luteal phase level of 1.74 ± 0.46 ng/ml (n = 10).

Fortunately, 17α hydroxyprogesterone is extracted by petrol ether to a much lesser extent compared to progesterone. For example, Johansson (1969), in studying the recovery of tritiated steroids from plasma by extraction with petrol ether, found that only 14% of the 17α hydroxy--progesterone was extracted, compared to 85.5% recovery of progesterone. The same worker found that the extraction of 20α dihydroprogesterone was 64%, but this compound has a much lower affinity for binding sites on the CBG molecule compared to either progesterone or 17α hydroxy--progesterone.

From these considerations, it would seem likely that simple petrol ether extraction alone, together with the relative specificity of the CBG molecules' binding sites, would be sufficient to ensure the specificity of

the method for progesterone. To ensure that this were so, duplicate samples of plasma were obtained. One sample of each pair was assayed by the "rapid" method described above, while the other was assayed by a similar method, in which the additional separation procedure of thin-layer chromatography (TLC) was incorporated. The results were then compared.

Method for Thin-Layer Chromatography (TLC)

Petrol ether extracts of plasma were prepared as described above, but washing with water was not carried out. Instead, the petrol ether extracts were dried under a stream of nitrogen in a water bath maintained at $45^{o}C$. A precoated silica chromatography sheet was divided into 6 lanes, each 3 cm wide by scratching lines in the silica gel. The dried extracts were then spotted on the sheets, using 350 microlitres of chloroform, as described by Neill et al. (1967). The dried progesterone standard was spotted in the same way, on a separate, adjacent lane. The sheets were developed in diethyl ether: benzene (2:1) by ascending chromatography. The areas in each lane, corresponding to the standard progesterone, chromatographed simultaneously and located under ultraviolet light, were cut out and eluted with 2 ml of absolute methanol, into round bottomed glass test tubes. The eluates were then evaporated to dryness under nitrogen, and 0.5 ml of CBG-^{3}H corticosterone was added to each. The remainder of the assay was performed

as described above. This chromatographic step effectively separates progesterone from both 17α hydroxyprogesterone and 20 dihydroprogesterone (Neill et al., 1967). The progesterone values obtained when this TLC step was performed were corrected for procedural losses according to the recovery of tritiated progesterone from aliquots of plasma subjected simultaneously to identical extraction and chromatography.

Comparison of Values Determined With and Without TLC

Four duplicate samples of plasma were obtained from women during the luteal phase, when interference with the progesterone assay by 17α hydroxyprogesterone and 20α di--hydroprogesterone would be expected to be maximal. One sample from each pair was assayed by the rapid method (omitting TLC), while the other sample was assayed including the TLC separation step. The results are shown below.

	Progesterone (ng/ml) with TLC	Progesterone (ng/ml) without TLC
	20.7	20.0
	6.0	7.6
	13.8	16.2
	20.4	20.5
Mean	15.2	16.1

An analysis of variance showed that there was no statistically significant difference between the mean values when progesterone was assayed with or without TLC (see statistical appendix). Therefore, all progesterone assays on non-pregnancy plasma were performed without the laborious step of TLC separation.

In pregnancy, however, there are greatly increased plasma levels of progesterone (and presumably, of its metabolites). Accordingly, it was considered that in measuring progesterone in pregnancy plasma, TLC was required to ensure that only progesterone would be allowed to displace 1,2-^{3}H corticosterone from CBG. Thus, all pregnancy plasmas were assayed using the TLC step.

B. THE MEASUREMENT OF OESTRADIOL IN PLASMA

Introduction

The pattern of urinary excretion of oestrone, oestriol and oestradiol during the normal menstrual cycle has been studied extensively (e.g. Brown, 1955; Ittrich, 1960; Loraine and Bell, 1963; Nocke and Breuer, 1963). Although the reliability of chemical methods of measuring urinary oestrogens had been established, there was clearly a need for an accurate method for measuring oestrogens in blood.

Svendsen and Sorensen (1964) described a double-isotope method for the measurement of "free" (i.e., non-conjugated) oestrone and oestradiol in plasma, but their method lacked adequate sensitivity, except to assay plasma oestrogen levels at the time of ovulation. Subsequently, Baird (1968) described an improved double-isotope derivative technique using ^{35}S-pipsyl chloride. This method appears to have adequate sensitivity and accuracy to allow the accurate measurement of free oestrone and oestradiol in the plasma of non-pregnant women. This method is, however, laborious, and requires up to 50 ml of blood for each assay, rendering daily blood sampling impracticable.

The development of methods for assaying oestradiol in plasma, based on the competitive protein-binding principle, has been a major advance. These newer assay techniques meet the requirements of accuracy, specificity and reliability, and require much smaller volumes of plasma.

The technique of oestradiol assay used in this study is based on the observation that oestradiol is bound specifically to uterine macromolecules (Talwar, Segal and Evans, 1964). This property has been demonstrated in several species, including the sheep and rabbit. Korenman et al. (1969) have described a competitive protein-binding radio--assay system for measuring oestradiol in human plasma. Their method utilises the oestradiol-binding properties of rabbit uterine macromolecules. Shutt (1969) has developed a method for assaying plasma oestradiol in which the binding macromolecules are obtained from sheep uteri. Subsequently, Dufau et al. (1970) developed an assay system using oestradiol-binding protein obtained from women in late pregnancy.

The method used in these studies was based on the original technique described by Shutt (1969), modified by the use of alumina column chromatography in place of paper chromato--graphy for the separation of oestradiol from oestrone. This modification, together with greater dilution of the

sheep uterine cytosol, resulted in a satisfactory reduction of blank values which were a problem in the original method. Basic data concerning the accuracy, specificity and reproducibility of the modified assay have been provided by Shutt and Cox (1971).

Method of Assay for Free Oestradiol in Plasma

A. MATERIALS

Aqueous solutions: Glass distilled water was used for all aqueous solutions.

Bicarbonate buffer (pH 9.9): $NaHCO_3$ and 2N NaOH were mixed in the ratio of 4.5:1

Binding system: Uteri from ovariectomised ewes were homogenised for 1 minute in 1-2 volumes of a cold sucrose (0.25M) and calcium chloride (3mM) solution, in a Servall stainless steel homogeniser. The homogenate was then centrifuged at 10,000g for 15 minutes to remove most of the cell debris, and then at 100,000g for 60 minutes. The soluble fraction obtained after centrifugation contained a macromolecular system which specifically bound oestradiol. The soluble fraction was stored in 2 ml aliquots at $-10^{\circ}C$ until use. It was found to remain stable for at least 4 months.

Chromatography was performed using specially constructed glass columns approximately 30 cm high, fitted with an expanded upper portion. The internal diameter of these columns measured 4-5 mm. Aluminium oxide powder (neutral, Woelm) was dried by heating overnight at 80°C, then hydrated to 4.5% by adding distilled water.

Dextran-coated charcoal suspension: Equal volumes of a 0.5G/100 ml solution of Dextran T 70 (Pharmacia, Uppsala) and a 5G/100 ml suspension of charcoal (Norit A, Pfanstiehl, Illinois) made up in 0.04M phosphate buffer, pH 7.2, were mixed together, stored at 4°C, and diluted 1:10 with the same buffer before use.

EDTA-Tris-HCl buffer: This buffer was prepared by adding EDTA (0.001M), sucrose (0.25M), Tris (0.01M) and HCl, and adjusting the pH to 7.2.

Oestradiol 2,4,6,7-^{3}H, specific activity 80,000-100,000mCi/mM was obtained from The Radiochemical Centre, Amersham, and diluted to provide two solutions of the isotope:

(a) Oestradiol-^{3}H in N saline, approximately 5,000 dpm per 0.1 ml, used for addition to plasma samples for the calculation of procedural losses.

(b) Oestradiol-^{3}H in ethanol, approximately 50,000 dpm per 0.2 ml, for use in the competitive protein-binding step.

Scintillation fluid: Toluene containing 0.3% p-diphenyl-benzene and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene.

Solvents were all of analytical grade and were distilled once before use.

B. METHODS

1. Extraction:

The extraction of plasma with ethyl acetate was performed using a specially constructed battery of 12 glass extraction vessels. This apparatus (known as the Paton-Brown partitioning extractor) has been described by Brown et al. (1968). Ten plasma samples of 4 ml, together with one "control" consisting of 200 pg of oestradiol added to 4 ml of distilled water, and one blank of 4 ml distilled water, were extracted with ethyl acetate simultaneously. Before extraction, 0.1 ml of ^{3}H-oestradiol in saline was added to each of the plasma samples and to the control sample, in order to estimate procedural losses. The 12 samples were then all extracted together with an equal volume (8 ml) of ethyl acetate for each.

The ethyl acetate extracts of plasma were washed once with 1 ml bicarbonate buffer (pH 9.9), then washed to neutrality with 2 washings using 2 ml distilled water. The ethyl acetate was evaporated off using a Buchi rotary evaporator, and the dried extracts taken up in 10 ml of 70% methanol, then transferred to glass centrifuge tubes. The aqueous methanol extracts were allowed to stand overnight at -10°C. The following day, the precipitated fat was spun down in a refrigerated centrifuge at -15°C for 45 minutes at 3,000 rpm.

The supernatant was transferred to glass round bottomed flasks (capacity 100 ml), and evaporated to dryness using the Buchi evaporator, equipped with a water bath maintained at 45°C. After evaporation to complete dryness, the oestrogens were taken up in 3 ml benzene by careful warming and rotation of each flask. Once taken up in benzene, the oestrogens remained stable for several days.

2. Chromatography

Aluminium oxide, 0.3G (± 0.05G) was weighed out in individual vials, and then packed into each glass column by adding the powder to the column filled with water-saturated n-hexane. After settling, the alumina formed a column approximately 15 mm high. The upper surface of the

powder was carefully levelled, and a small quantity of acid-washed sand was added, in order to prevent physical disruption of the upper surface of the alumina.

The oestrogen-containing benzene solution (3 ml) was then added to the top of the column; the flask was rinsed with a further 2 ml of benzene, which in turn was added to the column. The benzene solvent was allowed to run to waste. Elution of the oestrogens remaining on the column was carried out in two stages:

(a) "Oestrone fraction": Six ml of 1% ethanol/n-hexane were added after the benzene had run through. This fraction contained most of the oestrone present (Shutt and Cox, 1971). It was discarded.

(b) "Oestradiol fraction": After the above fraction had been allowed to run to waste, each column was eluted with 5 ml of 4% ethanol/n-hexane, and the eluate was collected in small glass test tubes. This fraction contained most of the oestradiol present, as will be demonstrated below.

After use, the alumina and sand were removed from the columns by rinsing with ethanol and n-hexane.

3. Estimation of Recovery of Oestradiol

One ml (i.e., 20%) of the "oestradiol fraction" was pipetted into a glass vial, and the ethanol/n-hexane solvent evaporated off under nitrogen. Scintillation fluid, 8 ml, was then added to the dry vial, which was capped, and together with duplicate vials containing 0.1 ml of the ^{3}H-oestradiol in saline added at the beginning of the assay, were counted in a Packard Liquid Scintillation Spectrometer. The counts obtained from the 1 ml portion of the "oestradiol fraction" were multiplied 5 times, to obtain the number of counts expected from the whole oestradiol fraction. The percentage recovery for each of the samples was calculated individually from the ratio of counts obtained from the oestradiol fraction to the counts obtained from the vials to which 0.1 ml of ^{3}H-oestradiol in saline had been added at the beginning of the assay.

$$\% \text{ Recovery} = \frac{\text{Counts from 1 ml oestradiol fraction}}{\text{Counts from 0.1 ml } ^{3}\text{H-oestradiol}} \times \frac{5}{1} \times \frac{100}{1}$$

4. Competitive Protein-Binding Step

To each tube containing 4 ml of 4% ethanol/n-hexane (the remainder of the "oestradiol fraction"), and to each pair of tubes containing oestradiol standards in 0.2 ml of ethanol, were added 0.2 ml of ^{3}H-oestradiol in ethanol (approximately 50,000 dpm). Eluates and standards were then

evaporated to dryness under a gentle stream of nitrogen, while the tubes were immersed in a water bath maintained at 45°C.

A 2 ml aliquot of the uterine cytosol preparation was thawed gently, and diluted with EDTA-Tris-HCl buffer to 20 ml. To each dried tube, 0.3 ml of this diluted solution was added, and each tube was carefully rotated by hand to ensure complete moistening of its walls with the uterine extract. The tubes were then incubated for one hour at 25°C. At the end of this time, 1 ml of dextran-coated charcoal suspension was added to each tube, in order to remove the free (displaced) tritiated oestradiol. The contents of each tube were mixed mechanically, then centrifuged for 20 minutes at 3,000 r.p.m. The clear supernatant was decanted quickly into glass vials containing 8 ml of scintillation fluid. After capping the vials and cooling for at least one half hour, radiation counting was performed. Duplicate 5 minute counts were obtained for all samples (plasma extracts, standards, and internal recoveries).

The reduction in the protein-bound ^{3}H-oestradiol was used to estimate the oestradiol present in the plasma extracts, by referring to the standard curve.

Preparation of Standard Curve

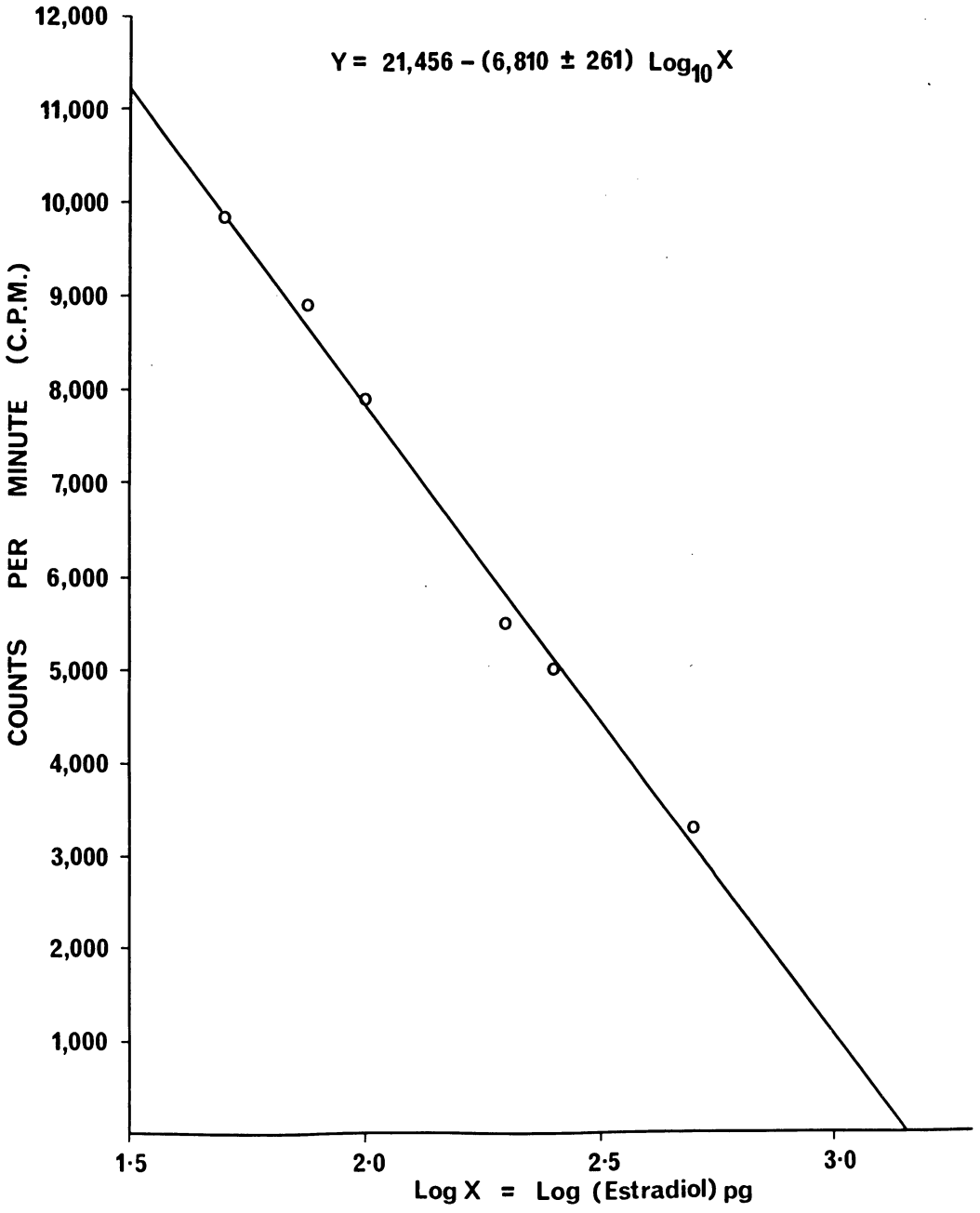
Oestradiol (Ikapharm, Israel) was dissolved in redistilled AR ethanol to make a solution of 1 mg/ml. From this stock solution, further dilutions with ethanol were made to prepare standard solutions containing 0, 10, 20, 50, 75, 100, 200, 250, 500, and 1,000 pg of oestradiol in 0.2 ml ethanol. These "working standards" were stored at $4^{o}C$, and were replaced at two-monthly intervals. A standard curve was prepared with each oestradiol assay. For routine purposes, duplicate 0.2 ml aliquots of each of the standard solutions were used. The standard curve was constructed by plotting the radioactivity of bound oestradiol against the log of the mass of unlabelled oestradiol added to the system.

A representative standard curve is shown, together with the data from which it has been constructed. To prepare this standard curve, quadruplicate estimations were performed to obtain each point. Within the range of 50-500 pg, the relationship of radioactivity of bound oestradiol to the log unlabelled oestradiol was found to be linear. Above and below this range, the addition of unlabelled oestradiol to the system caused relatively little displacement of bound tritiated oestradiol. Therefore, the volume of plasma was selected in order that the value might fall within this range of maximum sensitivity.

Table 4.4 Data Used in Construction of Standard Curve

Oestradiol (pg)	CPM	Mean CPM
0	8,617	11,461
	12,644	
	12,293	
	12,291	
10	10,510	11,219
	11,353	
	11,690	
	11,324	
20	10,573	10,489
	10,934	
	10,180	
50	10,229	9,858
	10,495	
	9,298	
	9,411	
75	8,439	8,878
	8,842	
	8,971	
	9,259	
100	8,176	7,890
	7,431	
	7,855	
	8,101	

Oestradiol (pg)	CPM	Mean CPM
200	5,517	5,493
	5,544	
	5,409	
	5,503	
250	4,212	4,961
	5,213	
	5,195	
	5,226	
500	3,388	3,319
	3,324	
	3,243	
	3,323	
1,000	1,783	1,824
	1,892	
	1,726	
	1,896	


Y = 21,456 – (6,810 ± 261) Log10 X
12,000
11,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
COUNTS PER MINUTE (C.P.M.)
1·5
2·0
2·5
3·0
Log X = Log (Estradiol) pg

Evaluation of the Method

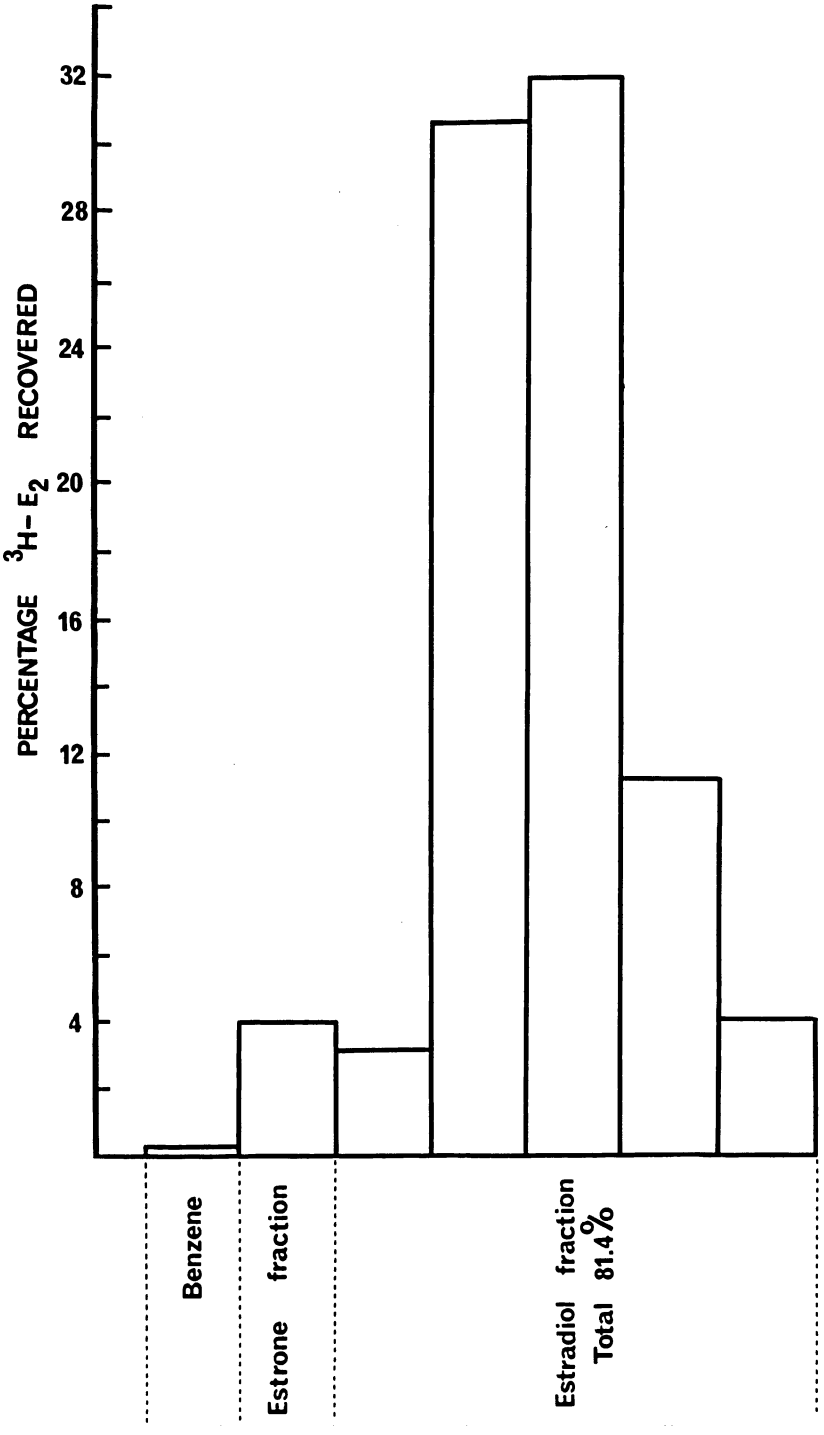
The method for the assay of oestradiol in plasma described here has been examined in detail by Shutt and Cox (1971), and found to meet the necessary requirements for specificity, accuracy, and reproducibility. Some additional data are presented to confirm the accuracy and reproducibility of the method, as used in the Prince Henry Hospital laboratory.

Recovery

The recovery of oestradiol was calculated for each sample as described. The range of recoveries was 30-65%. For example, in one assay involving 30 samples, the mean recovery was 44 $\pm$ 5.9% (n = 30). The overall recovery was influenced to a large degree by losses during the chromatography step, and in this, the state of hydration of the alumina, and the ratio of ethanol: n-hexane were major factors.The highest recoveries were obtained using 4.5% hydrated alumina, and 4% ethanol/n-hexane for elution of the "oestradiol fraction".

To investigate further the loss of oestradiol during chromatography, duplicate 0.1 ml aliquots of ^{3}H-oestradiol in ethanol (50,000 dpm) were added directly to vials, evap-orated, and counted. Similar duplicate aliquots were evaporated in round bottomed flasks, taken up in benzene,

RECOVERY OF ESTRADIOL FROM COLUMNS



and carried through the usual chromatographic procedure as described above. However, on this occasion, the benzene fraction and the oestrone fraction were both collected, and the oestradiol fraction was collected as 5 successive 1 ml fractions. The organic solvents were evaporated, and scintillation fluid was added to each of the vials. The percentage recovery of the original radioactivity in each of the fractions, is shown in the histogram. These figures show that on this occasion, using alumina with 4.5% hydration, and eluting the oestradiol fraction with 4% ethanol/n-hexane, the total recovery of oestradiol in the oestradiol total eluate of 5 ml was 81.4%.

Precision

The lower limit of sensitivity of the method was set by the value of the blank. For routine assays, the blank was provided by 4 ml of distilled water extracted with the control and plasma samples. Initially, blank values were high (over 100 pg), but repeated assaying using the same glassware and equipment resulted in a rapid reduction of the blank to 10-50 pg. During the studies reported here, an assay was repeated if the blank value exceeded 50 pg.

To check the accuracy of the method, known amounts of oestradiol were added to 4 ml of distilled water, and assayed.

Amount of oestradiol added to dist. water (picograms)	Values obtained by assay (pg)		Number of determinations
	Mean	S.D.	
200	207	24.8	8
250	251	25.8	21

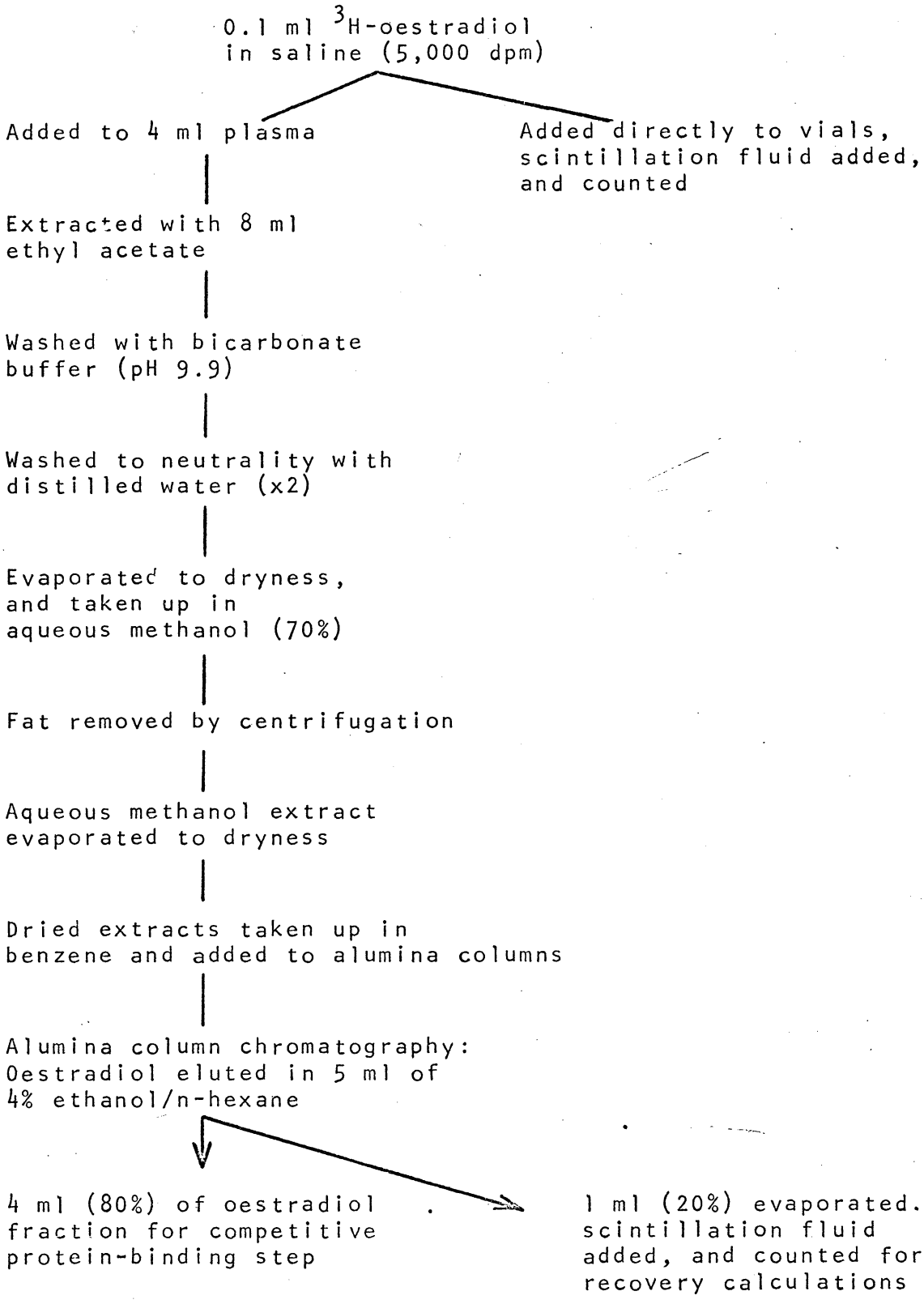
Reliability

The variation in values obtained in two different laboratories was investigated, by obtaining duplicate plasma samples from 6 normal, menstruating women. Two samples in duplicate were obtained: the first was taken on the seventh day of the cycle (follicular phase plasma), and the second on the twenty-first day (luteal phase plasma). Single assays using 4 ml of plasma were performed by the author in the Prince Henry Hospital laboratory, while duplicate assays using 2 ml of plasma were performed in the Veterinary Research Laboratories of the CSIRO, Prospect, N.S.W. A comparison of results is shown in Table 5. Analysis of variance shows that there is no significant difference in values obtained between the two laboratories (see statistical appendix).

Table 5 - Plasma Oestradiol Assay: Comparison of Results obtained in two different laboratories

Patient	Prince Henry Hospital		CSIRO, Prospect	
	Day 7	Day 21	Day 7	Day 21
A	11.3	19.1	6.4	13.6
B	4.4	20.1	5.4	20.3
C	4.7	11.3	5.2	10.7
D	2.7	5.9	3.3	6.3
E	7.4	9.2	6.3	6.8
F	8.3	11.2	8.2	19.0
Mean	6.4	12.8	5.8	12.8

SYNOPSIS OF OESTRADIOL ASSAY METHOD



4 ml of 4% ethanol/n-hexane (i.e., 80% of oestradiol fraction[11])

Duplicate pairs of oestradiol standards in 0.2 ml of ethanol

0.2 ml of ^{3}H-oestradiol (50,000 dpm) added to each tube containing eluate or standard

↓

Evaporation of organic solvents under nitrogen

↓

Addition of 0.3 ml diluted uterine extract

↓

Incubation for 1 hour at 25°C

↓

Addition of 1 ml dextran-coated charcoal suspension to remove displaced, labelled oestradiol

↓

Centrifuge, and decant supernatant into vials containing 8 ml scintillation fluid

↓

Cap, cool, and count radiation

CHAPTER 5

VARIATION IN DAILY LEVELS OF PROGESTERONE AND OESTRADIOL THROUGHOUT THE MENSTRUAL CYCLE IN NON-MIGRAINOUS WOMEN

5. VARIATION IN DAILY LEVELS OF PROGESTERONE AND OESTRADIOL THROUGHOUT THE MENSTRUAL CYCLE IN NON-MIGRAINOUS WOMEN

Procedure

Daily blood samples were obtained by venepuncture from 8 apparently healthy, regularly menstruating women, in order to study the normal pattern of variation in plasma concentrations of progesterone and oestradiol throughout the menstrual cycle. Progesterone assays were performed in duplicate on all plasma samples. Complete data concerning oestradiol levels were obtained in only 6 of these 8 women, owing to technical difficulties. Oestradiol concentrations were determined in single assays of 4 ml plasma. None of the women were taking any hormonal preparation. The methods used for progesterone and oestradiol assays have been described in previous chapters.

Results

A. PLASMA PROGESTERONE DETERMINATIONS

Five of the 8 women showed typical ovulatory patterns of progesterone concentrations. In these women, the levels remained low (less than 2 ng/ml) during menstruation and most of the follicular phase, and began to rise slightly in the pre-ovulatory period. Following ovulation in mid-cycle, there was a marked increase in plasma progesterone,

and high, though fluctuating levels were maintained until the premenstrual phase, when levels fell rapidly, until at the onset of menstruation, follicular phase levels had again been reached.

Plasma progesterone concentrations found in these 5 ovulatory cycles are shown in the accompanying graph and table. The progesterone values have been plotted around the onset of menstruation ("day 0"), since this phase of the cycle is the focal point of interest in the study of menstrual migraine.

Two women showed atypical patterns. In one, there was no significant rise in progesterone during the second half of the cycle, while in the other, the rise was small and transient. As there was no sustained rise in progesterone during the luteal phase, it is presumed that these cycles were anovular.

The remaining woman became pregnant during the cycle under study. It is of interest that the plasma progesterone concentration rose above the usual luteal phase level (less than 20 ng/ml) as early as the twenty third day of the cycle (i.e., 12 days after the ovulatory peak of oestradiol secretion). Since this woman had a regular 28-29 day cycle, it was possible to diagnose pregnancy before the expected date of menstruation.

TABLE 5.1 - Plasma concentrations of progesterone measured daily throughout the menstrual cycle in 5 normal, non-migrainous women.

Day of cycle	Plasma progesterone (ng/ml)				
	J.S.	B.W.	A.Z.	S.B.	A.B.
0	1.0	0.4	0.8	0.5	0.5
1	0.5	0.5	1.0	0.5	0.5
2	0.5	0.5	0.6	0.5	0.8
3	1.0	0.5	0.5	0.6	0.6
4	0.8	0.6	1.2	0.5	0.6
5	0.6	0.5	0.5	-	0.5
6	1.0	0.5	0.6	1.3	0.5
7	1.0	0.5	1.2	1.4	0.8
8	1.2	-	0.5	1.0	0.6
9	0.8	0.5	2.4	-	1.0
10	1.0	0.8	2.0	0.6	-
11	1.2	1.0	3.6	1.5	0.8
12	1.0	1.0	5.2	1.7	1.0
13	1.4	1.4	-	1.7	5.0
14	3.0	1.6	12.0	6.2	7.0
15	3.6	3.2	8.0	3.0	8.0
16	2.8	2.0	8.0	5.2	12.5
17	4.6	3.5	9.0	4.0	10.5
18	5.9	2.6	11.0	4.0	6.5
19	6.0	3.8	6.4	3.5	6.5
20	8.8	6.5	12.8	6.6	6.2
21	17.1	9.5	8.8	5.8	7.5
22	-	6.6	4.8	4.2	8.0
23	22.0	7.0	0.8	2.8	4.5
24	19.7	7.5		2.4	0.6
25	18.6	2.4		0.5	0.7
26	10.3	7.0		0.5	
27	2.0	3.0		0.5	
28	0.8	2.0			

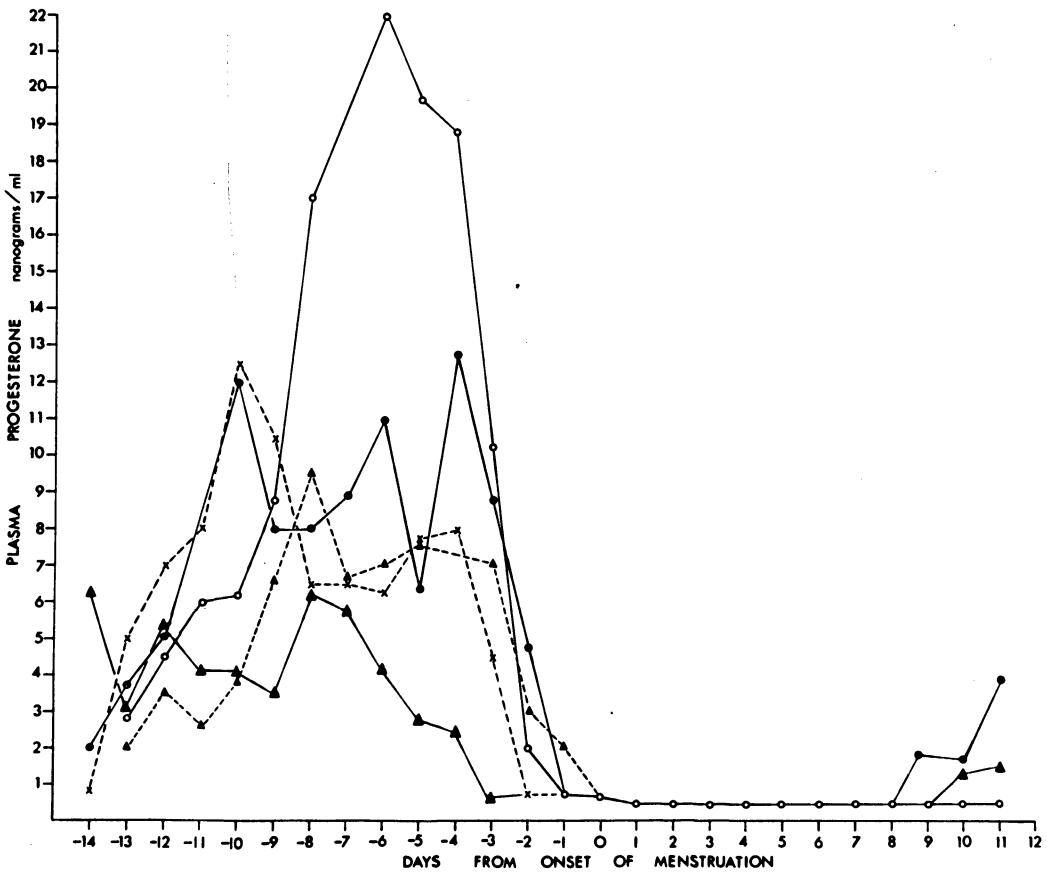


Figure 5.1 - Plasma Progesterone Concentrations measured daily throughout the menstrual cycle in 5 normal, non-migrainous women.

B. PLASMA OESTRADIOL DETERMINATIONS

In all of the 5 women studied, plasma oestradiol remained low (less than 10 ng/100 ml) during menstruation and the early part of the follicular phase. A marked peak then occurred toward mid-cycle, corresponding to the "ovulatory peak" in urinary oestrogen excretion (Brown, 1955) In 4 women, this peak occurred before or at mid-cycle, while in the remaining subject the peak was delayed, and was observed on the 17th day of a 22-day cycle. The latter subject failed to show any rise in plasma progesterone during the luteal phase, and was thought to have an anovulatory cycle (subject A.M.).

Although well-defined oestradiol peaks characterised the other women's cycles, marked individual variations were noted between peak oestradiol values (range 14.4 ng/100 ml to 31.6 ng/100 ml), and in the duration of the oestradiol peak.

Following the oestradiol peak, a rapid fall to follicular phase levels was noted in all subjects. Then, a secondary rise in oestradiol levels was observed. This took the form either of a sustained increase or "plateau" (e.g. subject M.J.), or of a series of intermittent peaks (e.g. subject B.W.). In none of the women did luteal phase

oestradiol levels exceed the ovulatory peak value. In the 4 normal, ovulatory cycles, a distinct rise in plasma oestradiol levels, compared to follicular phase values, was observed during the luteal phase. This persisted until a few days before menstruation.

During the premenstrual phase, a rapid decline in oestradiol levels was observed in all women. In general, this was followed closely by the decline in plasma levels of progesterone, suggesting that the fall in levels of both hormones was a consequence of senescence of the corpus luteum.

TABLE 5.2 - Plasma Concentrations of Progesterone and Oestradiol measured daily during the Menstrual Cycle in Four Normal Women.

Figures 5.2 to 5.5 have been constructed from these data.

Day of cycle	Mrs. B.W. Progesterone ng/ml	Mrs. B.W. Oestradiol ng/100 ml	Mrs. E.K. Progesterone ng/ml	Mrs. E.K. Oestradiol ng/100 ml
0	-Progesterone values at limit of sensitivity-	2.8	-Values at limit of sensitivity-	3.3
1		3.0		5.6
2		2.7		6.7
3		3.6		-
4		3.8		13.0
5		4.0		-
6		6.0		25.6
7		1.8		30.3
8		-	1.1	31.6
9		-	1.6	19.0
10		-	2.6	7.8
11	1.0	7.0	3.8	7.0
12	1.0	16.6	7.2	7.2
13	1.4	21.5	9.5	11.4
14	1.6	13.8	14.0	15.5
15	3.2	6.8	-	-
16	2.0	11.3	14.1	11.6
17	3.5	5.5	-	-
18	2.6	-	9.0	8.3
19	3.8	-	10.0	13.0
20	6.5	9.5	7.8	8.4
21	9.5	6.5	4.6	8.2
22	6.6	8.5	1.9	4.0
23	7.0	-		
24	7.5	10.0		
25	7.3	9.4		
26	3.0	6.2		
27	2.0	3.1		

	Mrs. S.B.		Mrs. A.B.	
Day of cycle	Progesterone ng/ml	Oestradiol ng/100 ml	Progesterone ng/ml	Oestradiol ng/100 ml
0	0.5	0.9	0.5	3.2
1	0.5	0.9	0.5	7.3
2	0.5	-	0.8	-
3	0.6	1.4	0.6	5.2
4	0.5	2.5	0.6	-
5	-	1.6	0.5	12.0
6	1.3	2.2	0.5	22.8
7	1.4	2.4	0.8	12.3
8	1.0	4.4	0.6	9.5
9	-	3.6	1.0	7.5
10	0.6	4.8	-	-
11	1.5	11.4	0.8	4.9
12	1.7	14.4	1.0	9.1
13	1.7	8.2	5.0	15.1
14	6.2	1.6	7.0	19.5
15	3.0	4.1	8.0	21.9
16	5.2	3.7	12.5	14.3
17	4.0	5.2	10.5	11.5
18	4.0	5.8	6.5	-
19	3.5	5.0	6.5	5.2
20	6.6	6.9	6.2	-
21	5.8	6.7	7.5	7.0
22	4.2	8.7	8.0	6.3
23	2.8	6.2	4.5	7.9
24	2.4	4.1	0.6	8.5
25	0.5	2.0	0.7	3.1
26	0.5	1.3		
27	0.5	-		

Figure 5.2

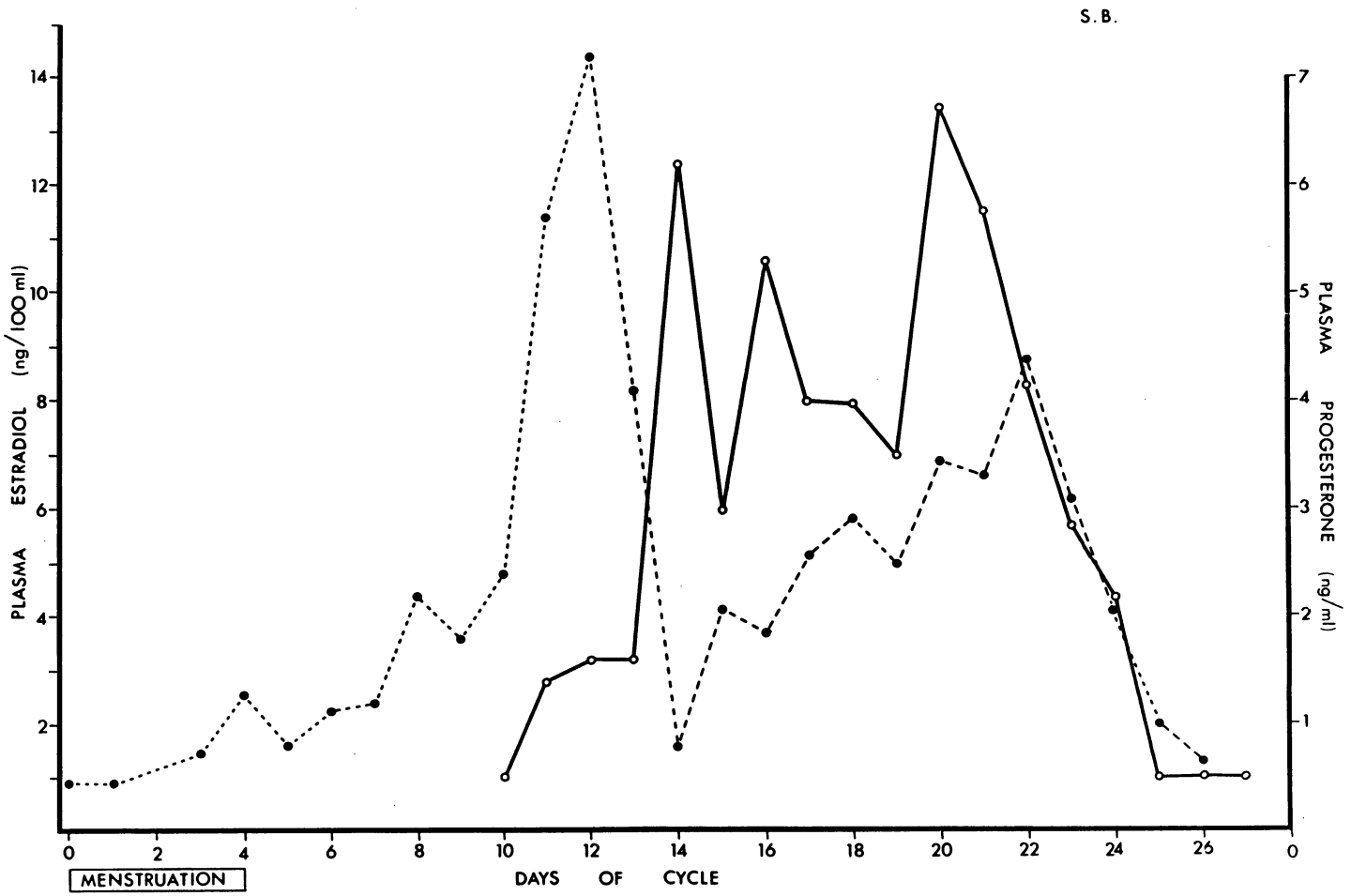
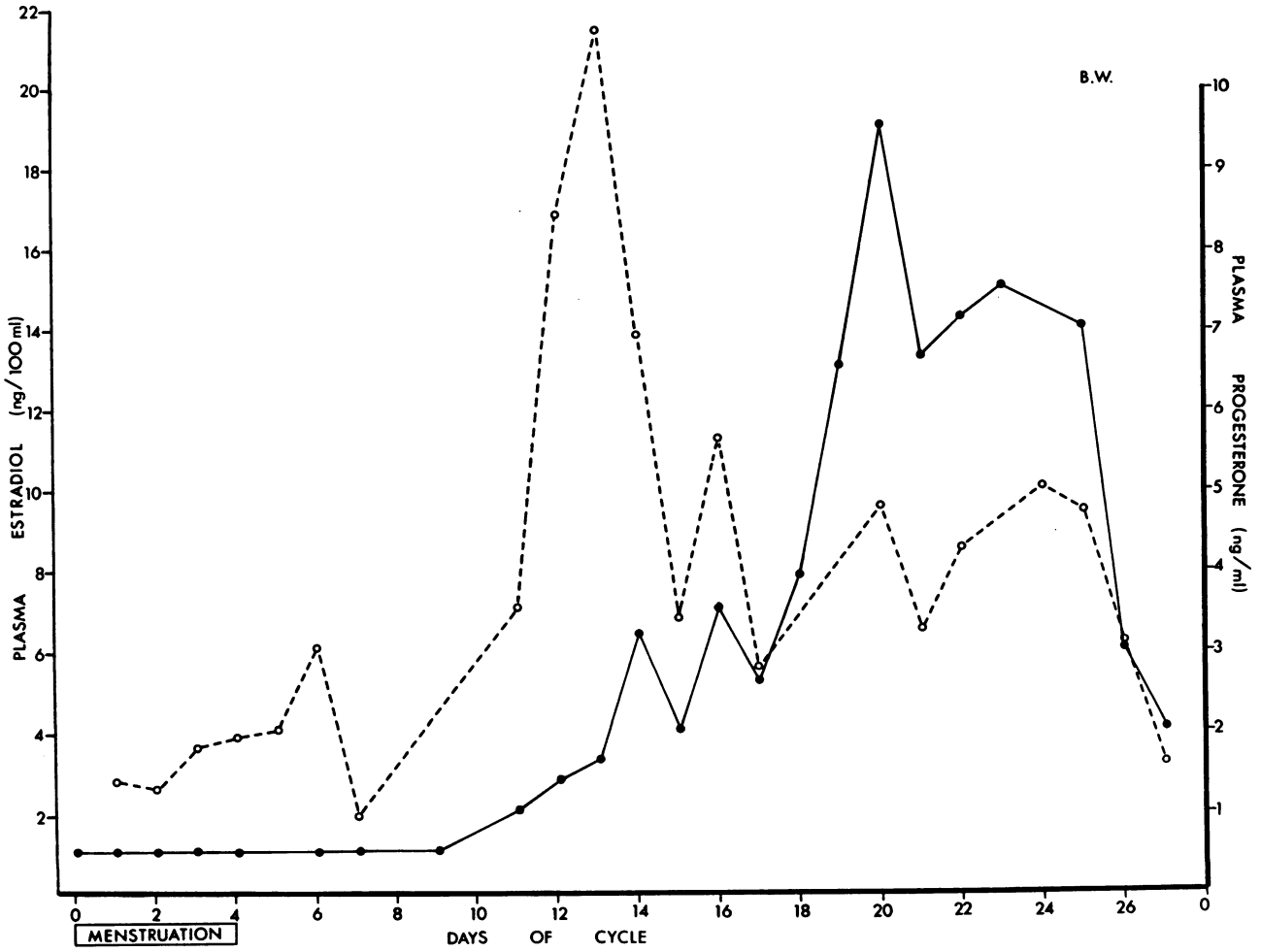

S.B.
PLASMA ESTRADIOL (ng/100 ml)
PLASMA PROGESTERONE (ng/ml)
14
12
10
8
6
4
2
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
0
MENSTRUATION
DAYS OF CYCLE

Figure 5.3


B.W.
PLASMA ESTRADIOL (ng/100 ml)
PLASMA PROGESTERONE (ng/ml)
22
20
18
16
14
12
10
8
6
4
2
10
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
0
MENSTRUATION
DAYS OF CYCLE

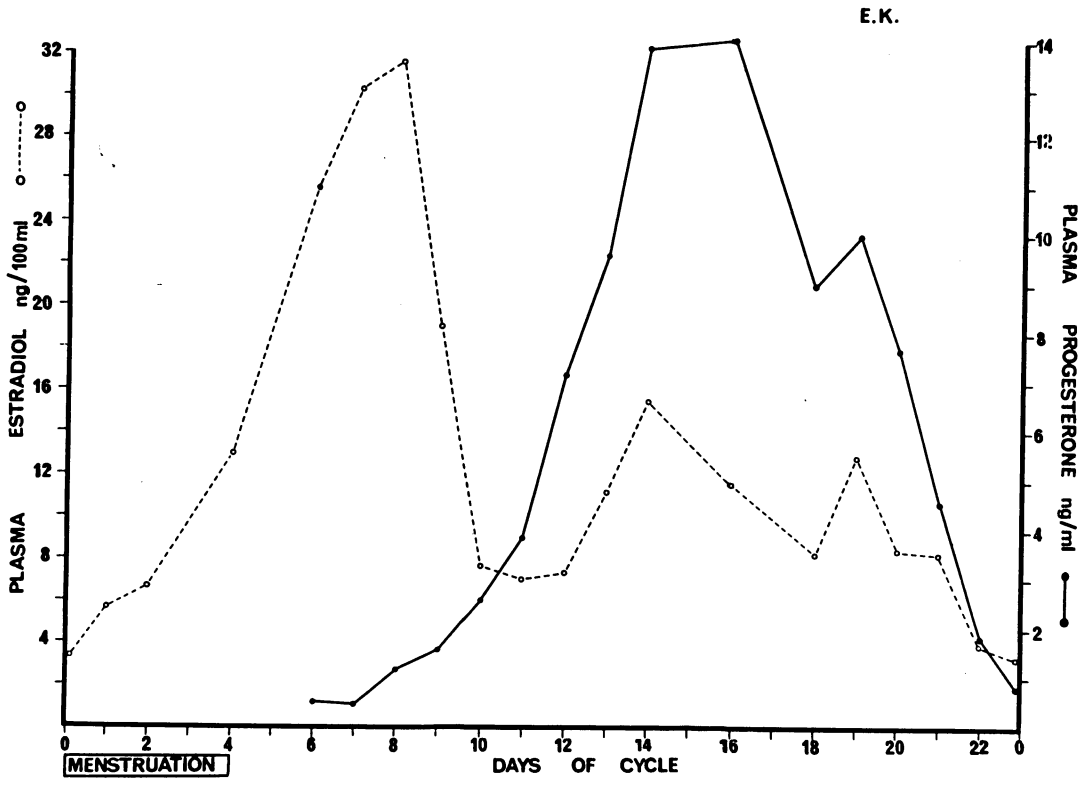

E.K.
PLASMA ESTRADIOL ng/100ml
PLASMA PROGESTERONE ng/ml
32
28
24
20
16
12
8
4
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
22
0
MENSTRUATION
DAYS OF CYCLE

Figure 5.4

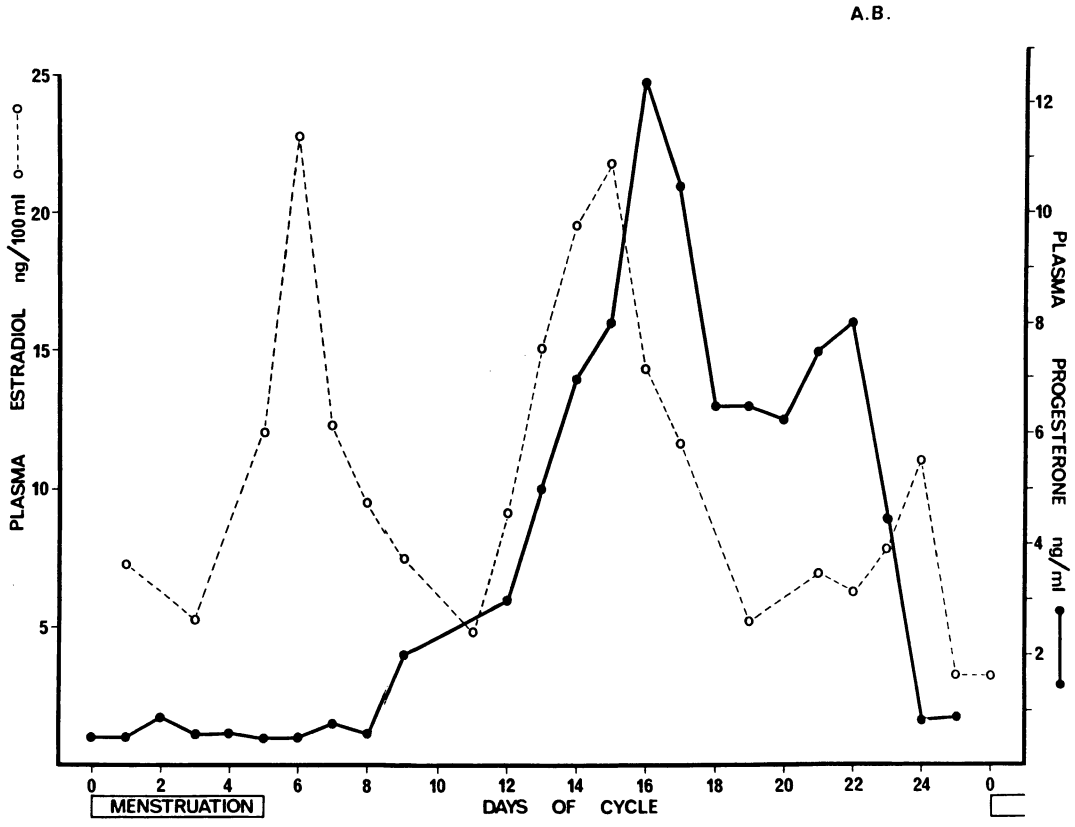

A.B.
PLASMA ESTRADIOL ng/100ml
PLASMA PROGESTERONE ng/ml
25
20
15
10
5
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24
0
MENSTRUATION
DAYS OF CYCLE

Figure 5.5

Table 5.3 - Plasma Concentrations of Oestradiol throughout an Anovulatory Menstrual Cycle*

Day of cycle	Plasma Oestradiol (ng/100 ml)
0	0.8
1	1.0
2	2.8
3	2.4
4	2.0
5	1.3
6	-
7	1.4
8	2.4
9	2.7
10	2.0
11	1.5
12	2.5
13	3.9
14	-
15	4.7
16	6.8
17	14.6
18	13.7
19	7.0
20	3.1
21	3.9
22	2.0

*Since plasma progesterone values did not exceed 2 ng/ml throughout the cycle, they have not been shown above.

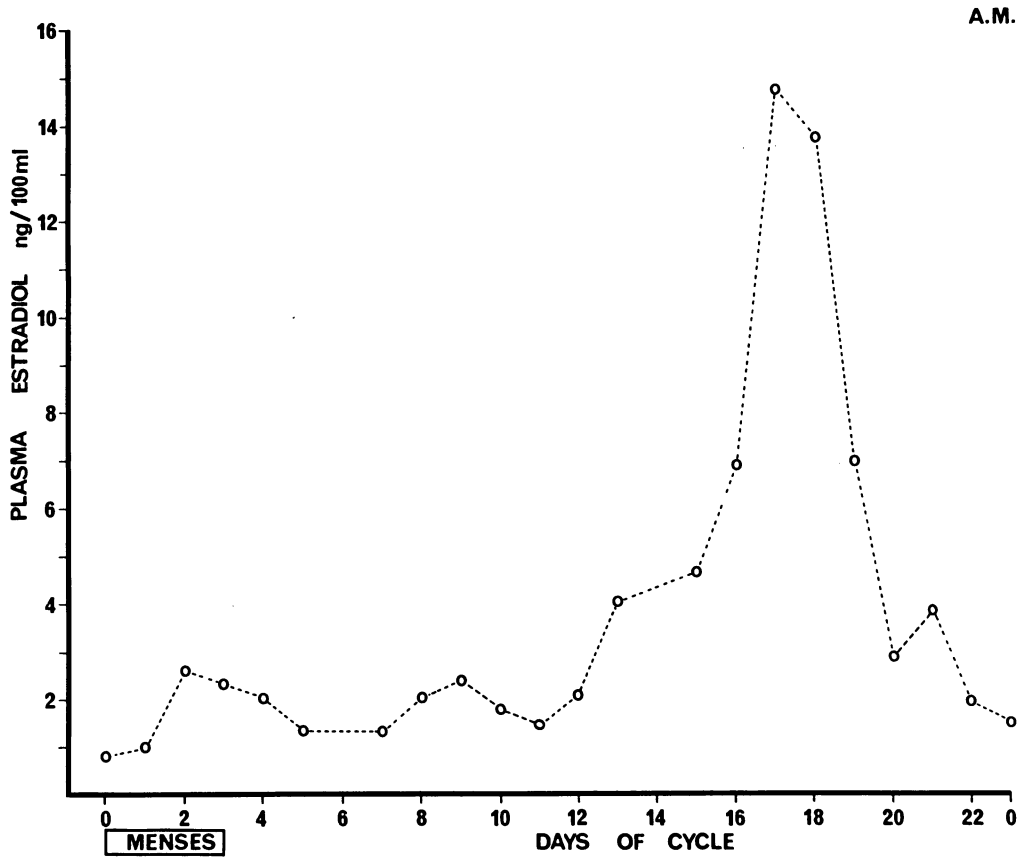


Figure 5.6 - Daily plasma oestradiol levels during an anovulatory cycle.

TABLE 5.4: Plasma Concentrations of Progesterone and Oestradiol during a cycle in which Fertilisation occurred.

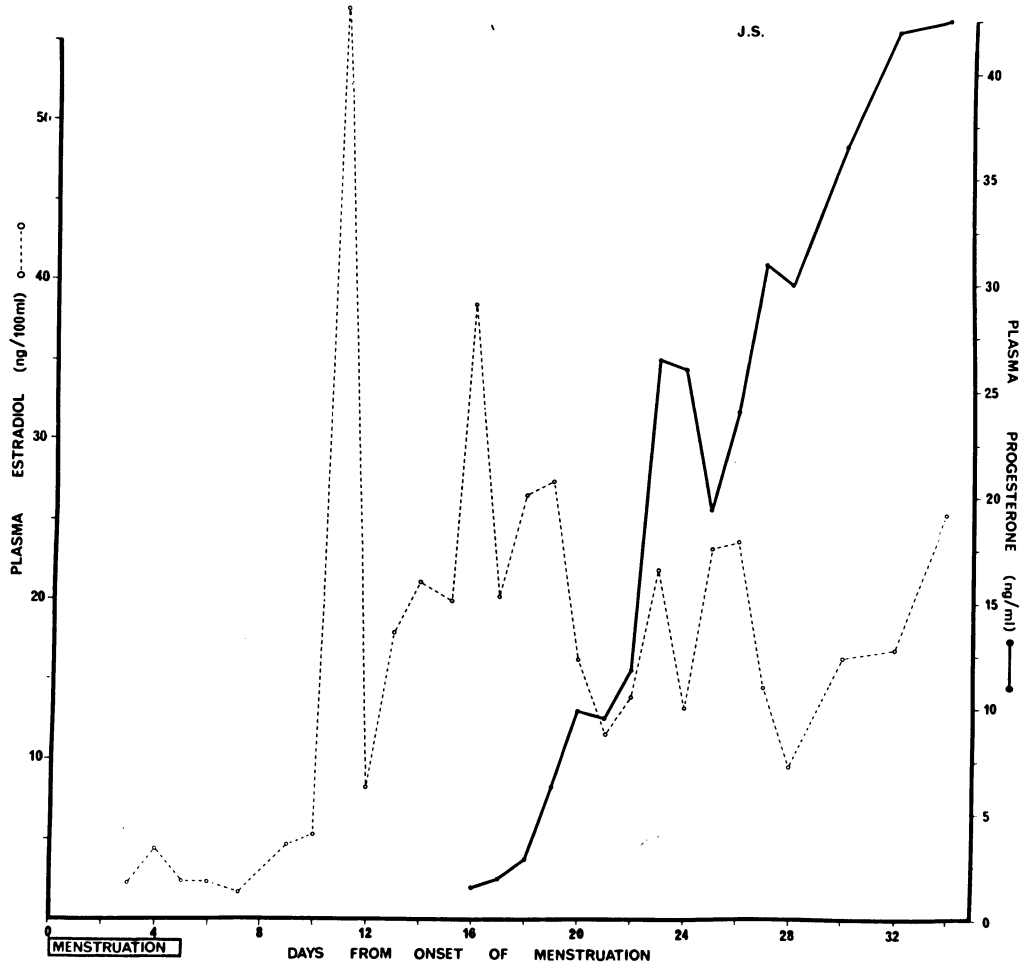


Figure 5.7 - Daily plasma concentrations of progesterone and oestradiol during a cycle in which fertilisation occurred.

Day of cycle	Progesterone (ng/ml)	Oestradiol (ng/100 ml)
0		-
1		-
2		-
3		1.7
4		4.3
5		1.9
6		1.8
7		1.4
8		-
9		4.7
10		5.6
11		57.2
12		8.3
13		18.2
14		21.1
15	-Progesterone values at limit of sensitivity-	20.0
16	1.4	38.6
17	1.8	20.4
18	2.8	26.8
19	6.2	27.5
20	9.8	16.1
21	9.6	11.7
22	11.8	14.1
23	26.2	22.2
24	26.0	13.2
25	19.5	23.4
26	24.0	23.5
27	31.0	14.6
28	30.0	9.6
30	34.0	16.4
32	42.0	17.0
34	42.5	25.6

Discussion

The values for plasma progesterone concentrations obtained here agree well with those reported by earlier workers using chemical methods (e.g. Riondel et al., 1965), and with those of more recent workers using competitive protein-binding methods of assay (e.g. Neill et al., 1967; Yoshimi and Lipsett, 1968; Johansson, 1969).

The largest study of normal plasma progesterone patterns to date has been that of Johansson (1969), who suggested these criteria for the normal menstrual cycle:

(a) The plasma progesterone concentration should rise steadily immediately after the peak in urinary total oestrogen excretion, or the peak in plasma LH.

(b) The "plateau values" during the luteal phase should reach 10-20 ng/ml.

(c) The progesterone concentration should have fallen below 1 ng/ml when menstrual bleeding commences.

Excluding the woman who became pregnant; and allowing for variation between the sensitivities of Johansson's method and the present assay, it can be seen that 5 of the 7 women fulfilled these criteria.

Plasma oestradiol values obtained during the current study are similar to those reported by workers using assay systems based on double-isotope derivative methods (Baird and Guevara, 1969), radioimmunoassay (Abraham, 1969), and competitive protein-binding (Korenman et al., 1969; Corker et al., 1969; Dufau et al., 1970). The variable, but generally low levels of plasma oestradiol during menstruation and the early part of the follicular phase have been found by the last three groups of workers, and agree well with earlier studies of urinary oestrogen excretion throughout the cycle (Brown, Klopper and Loraine, 1958). In one of the cycles of 25 days, the oestradiol peak was observed as early as the sixth day. A similar early peak was observed in one of the cycles studied by Dufau et al. (1970).

It has been suggested that the first (pre-ovulatory) oestradiol peak acts as the stimulus for the release of LH, leading to ovulation. Recently, Vande Wiele et al. (1970) have shown that the intravenous administration of oestrogens to anovulatory women causes a LH peak to appear. These workers also reported data concenring plasma levels of oestradiol, progesterone, LH, and FSH measured throughout the menstrual cycle in 2 women. The patterns they found agree closely with those obtained in the present study.

The relationship between plasma levels of progesterone and oestradiol found in this study appears to support the hypothesis that the mid-cycle LH peak is evoked by the pre-ovulatory peak in oestradiol secretion. In none of the four "ovulatory" cycles did the plasma progesterone begin to rise substantially until the pre-ovulatory oestradiol peak had occurred. Although a small pre-ovulatory rise in plasma progesterone was observed in two of these cycles, this could be explained by the ability of the maturing follicle to secrete progesterone (Runnebaum and Zander, 1967), while the marked rise in progesterone following the oestradiol peak presumably arose from the secretion of the newly-formed corpus luteum. In the anovulatory cycle in which the oestradiol peak was delayed until the seventeenth day, there was no significant rise in plasma progesterone, and the menstrual bleeding which followed was evidently due to the withdrawal of oestrogens alone. The correct timing of the oestradiol peak early in the cycle would therefore appear to be of critical importance in the initiation of ovulation.

It is of interest that the plasma levels of progesterone and oestradiol declined more or less simultaneously during the premenstrual phase of the four normal cycles. This pattern is consistent with the view that both hormones are secreted principally by the corpus luteum, whose degener-ation results in falling levels of these hormones during the premenstrual phase. This pattern of simultaneous decline

in plasma levels of oestradiol and progesterone had been suggested by earlier studies of the urinary excretion of oestrogens and pregnanediol during the normal menstrual cycle (Brown et al., 1958).

During the cycle in which fertilisation occurred, an elevation of plasma progesterone to above usual luteal phase values became apparent 12 days after the oestradiol peak. In a similar study of plasma progesterone ab initio in pregnancy, Johansson (1969) found a plasma progesterone elevation above 20 ng/ml at 8 days after the peak in urinary oestrogen excretion. There is considerable evidence that the corpus luteum is the major source of the increased secretion of progesterone and oestradiol during the first 6-10 weeks of pregnancy (Yoshimi et al., 1969). The mechanism whereby the corpus luteum is informed that fertilisation has taken place appears to operate through the secretion of chorionic gonadotrophin (HCG), which appears as early as 12 days after the mid-cycle peak of LH. The main source of HCG during early pregnancy is the syncytiotrophoblast of the newly imbedded, fertilised ovum (Midgley and Pierce, 1963).

It is noteworthy that the early surge in progesterone during pregnancy was not accompanied by a simultaneous rise in plasma oestradiol, although previous data showed that the urinary excretion of oestriol may rise above the range

normally found during the luteal phase as early as 15-20 days after the mid-cycle peak in urinary oestrogen excretion (Loraine and Bell, 1963). The absence of a marked early rise in plasma oestradiol may therefore be the result of a relative insensitivity of the corpus luteum to the effect of HCG in stimulating oestradiol secretion, or to more efficient metabolism of an increased secretion of oestradiol in early pregnancy. It should be emphasised that the pregnancy which began in the cycle under study terminated in the fourth month by spontaneous abortion, so that the hormonal changes may not reflect those of a "normal" pregnancy.

SUMMARY

Daily plasma concentrations of progesterone and oestradiol were measured throughout the menstrual cycle in 6 apparently healthy, regularly-menstruating women. Four women showed typical "ovulatory" patterns; the fifth subject had an anovulatory cycle, while the remaining woman became pregnant, and subsequently aborted spontaneously during the fourth month.

During the 4 ovulatory cycles, the plasma progesterone rose sharply following the pre-ovulatory oestradiol peak. The premenstrual phase was characterised by a simultaneous

decline in plasma levels of both progesterone and oestradiol. In the subject who became pregnant during the cycle under study, the early surge in plasma progesterone was not accompanied by a similar rise in plasma oestradiol. The anovulatory cycle was associated with a delayed peak in oestra-diol levels, and an absence of significant elevation in plasma progesterone during the second half of the cycle.

Plasma progesterone concentrations were measured throughout the cycle in 2 further women, for whom plasma oestradiol estimations could not be obtained. One of these women showed a typical "ovulatory" pattern, while the other failed to show any sustained increase in progesterone during the luteal phase, suggesting that this cycle may have been anovular.

The significance of these hormone patterns is discussed in relation to the underlying ovarian physiology.

CHAPTER 6

DAILY VARIATION IN PLASMA LEVELS OF PROGESTERONE AND OESTRADIOL THROUGHOUT THE MENSTRUAL CYCLE IN WOMEN SUBJECT TO MENSTRUAL MIGRAINE

6. DAILY VARIATION IN PLASMA LEVELS OF PROGESTERONE AND OESTRADIOL THROUGHOUT THE MENSTRUAL CYCLE IN WOMEN SUBJECT TO REGULAR MENSTRUAL MIGRAINE

Introduction

The regular recurrence of migraine during the premenstrual or menstrual phases of the cycle suggests that withdrawal of sex hormones may play a significant role in the precipit--ation of migraine in some women. Since it has been shown that, toward the end of the cycle, plasma levels of progesterone and oestradiol usually decline simultaneously, it was consid--ered that migraine might be initiated by withdrawal of either progesterone, or oestradiol, or of both hormones.

As a preliminary step, it was felt necessary to measure plasma levels of progesterone and oestradiol throughout the menstrual cycle in a small number of women subject to regular, recurrent premenstrual or menstrual migraine, in order to confirm that the migraine did in fact commence during the phase of hormone withdrawal, and to compare hormone patterns of the migrainous women with those of normal, non-migrainous women described previously.

Subjects

Initially, 5 women were studied. Each suffered from regular premenstrual or menstrual migraine. The headaches were unilateral in 4 of the 5 women, and in all cases, the migraine attack was accompanied by nausea with or without vomiting, and some degree of photophobia. The duration of headaches ranged from 6 to 48 hours. In two subjects, prodromal visual symptoms (photopsia, fortification spectra) were prominent. In each case, there had been one severe attack each month, clearly related to menstruation, and recurring for at least 6 successive menstrual cycles immediately preceding the investigation.

Procedure

Blood was collected daily by venepuncture, as described. The plasma obtained was frozen at -10^{o}C until the cycle had been completed. Progesterone was then determined using duplicate 0.5-1.0 ml samples of plasma, and oestradiol was assayed using single 4 ml samples of plasma. All women were asked to record accurately the onset and duration of menstruation, and the day and time of onset of migraine, its duration, and any associated symptoms, such as nausea or visual disturbance. Progesterone determinations were obtained in all 5 women. Because of technical difficulties, data concerning oestradiol levels were obtained in only 3 subjects.

Results

Daily plasma progesterone values throughout the menstrual cycle in the 5 migrainous women are shown in Table 6.1 and Figure 6.1. In all subjects, the progesterone showed a clearly "ovulatory" pattern. Values were low during menstruation and the follicular phase, with a clear cut rise at or just after mid-cycle; sustained high levels were observed during the luteal phase, with a marked premenstrual decline. The mean of the peak progesterone concentrations in the migrainous group was 15.3 ± 2.21 ng/ml, which did not differ significantly from that of the non-migrainous group, mean 12.7 ± 5.95 ng/ml ($p > 0.10$).

All 5 migrainous women developed their usual headaches, one at the onset of the falling phase of progesterone levels, one during this phase, and 3 at its termination.

In the 3 women for whom plasma oestradiol data were available, normal "ovulatory" patterns of oestradiol concentrations were observed. Thus, there was an early preovulatory peak, followed by a rapid fall. Plasma levels of oestradiol then showed a variable secondary rise during the luteal phase, with a final fall during the premenstrual phase. In 2 women, the migraine began during the phase of oestradiol withdrawal, while in the remaining woman, migraine

commenced 2 days after the plasma oestradiol had fallen to its lowest level.

From these results, it was concluded that menstrual migraine showed at least a close temporal relationship to the phase of withdrawal of oestradiol and progesterone. Further studies were embarked upon in order to clarify the role of withdrawal of each hormone individually.

TABLE 6.1 - PLASMA PROGESTERONE CONCENTRATIONS (ng/ml) IN FIVE WOMEN SUBJECT TO REGULAR MENSTRUAL MIGRAINE

Days from onset of menstruation	M.G.	M.J.	E.Ki.	E. Ko.	J.C.
-14	3.7	8.8	4.0	2.7	
-13	9.2	11.1	12.0	2.8	
-12	10.2	15.0	11.3	3.5	
-11	15.0	14.7	11.5	4.1	
-10	14.0	14.5	15.2	4.7	
-9	11.8	14.1	16.8	5.9	
-8	13.5	12.0	18.2	7.7	
-7	12.5	11.2	15.4	12.2	
-6	11.8	10.2	10.0	9.0	15.3
-5	6.4	8.0	5.6	7.6	8.0
-4	7.0	7.0	3.2	8.5	12.0
-3	7.3	5.8	2.8	4.0	8.5
-2	6.0	6.5	2.5	0.8	4.0
-1	3.3	3.0	1.0	0.5	3.1
0	2.0	1.8	1.0	0.5	1.5

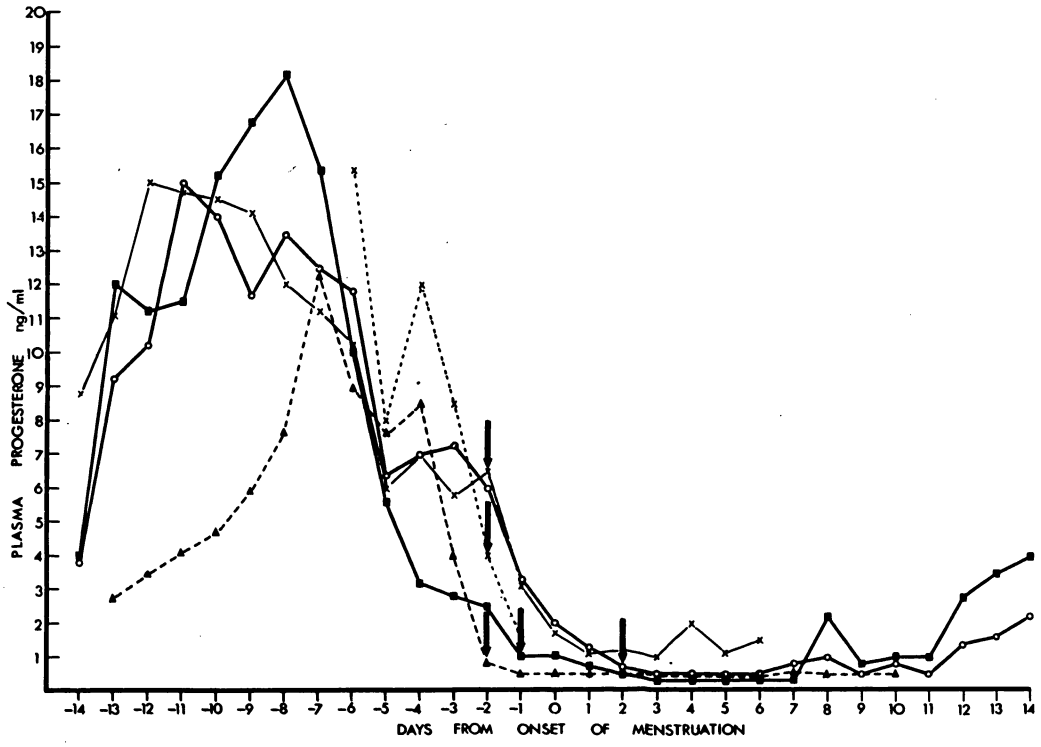


Figure 6.1 - Plasma Progesterone Concentrations In 5 women subject to Regular, Recurrent Menstrual Migraine. Vertical arrows Indicate the onset of migraine.

TABLE 6.2 - Plasma Progesterone and Oestradiol Levels during the menstrual cycle in 3 women subject to regular, recurrent menstrual migraine.

Figures 6.2 to 6.4 have been constructed from these data.

	Mrs. M.J.		Mrs. M.G.	
Day of cycle	Progesterone ng/ml	Oestradiol ng/100 ml	Progesterone ng/ml	Oestradiol ng/100 ml
0	1.8	1.0	2.0	2.8
1	0.8	-	1.3	4.5
2	0.8	8.1	1.0	4.0
3	0.8	-	1.0	4.4
4	0.7	12.7		
5	0.5	2.2		
6	1.2	5.7		
7	0.8	9.3	0.8	2.0
8	0.5	4.3	1.0	7.9
9	0.6	-	0.5	12.3
10	0.8	-	0.7	13.0
11	2.0	22.9	0.5	20.5
12	1.0	24.7	1.3	29.0
13	3.2	19.6	1.8	36.1
14	4.0	4.0	2.1	19.7
15	6.7	8.3	3.2	3.0
16	8.4	8.0	3.7	1.9
17	11.5	-	9.2	9.0
18	15.0	21.0	10.1	6.0
19	13.7	-	15.0	9.5
20	14.7	24.0	14.1	8.0
21	13,9	-	11.7	8.3
22	12.1	-	13.5	7.1
23	11.2	19.7	12.6	9.0
24	10.0	16.9	11.8	7.1
25	6.0	-	6.7	4.0
26	7.0	-	7.0	-
27	5.6	16.1	7.3	2.8
28	5.4	-	6.0	2.9
29	3.2	6.3	3.2	2.0

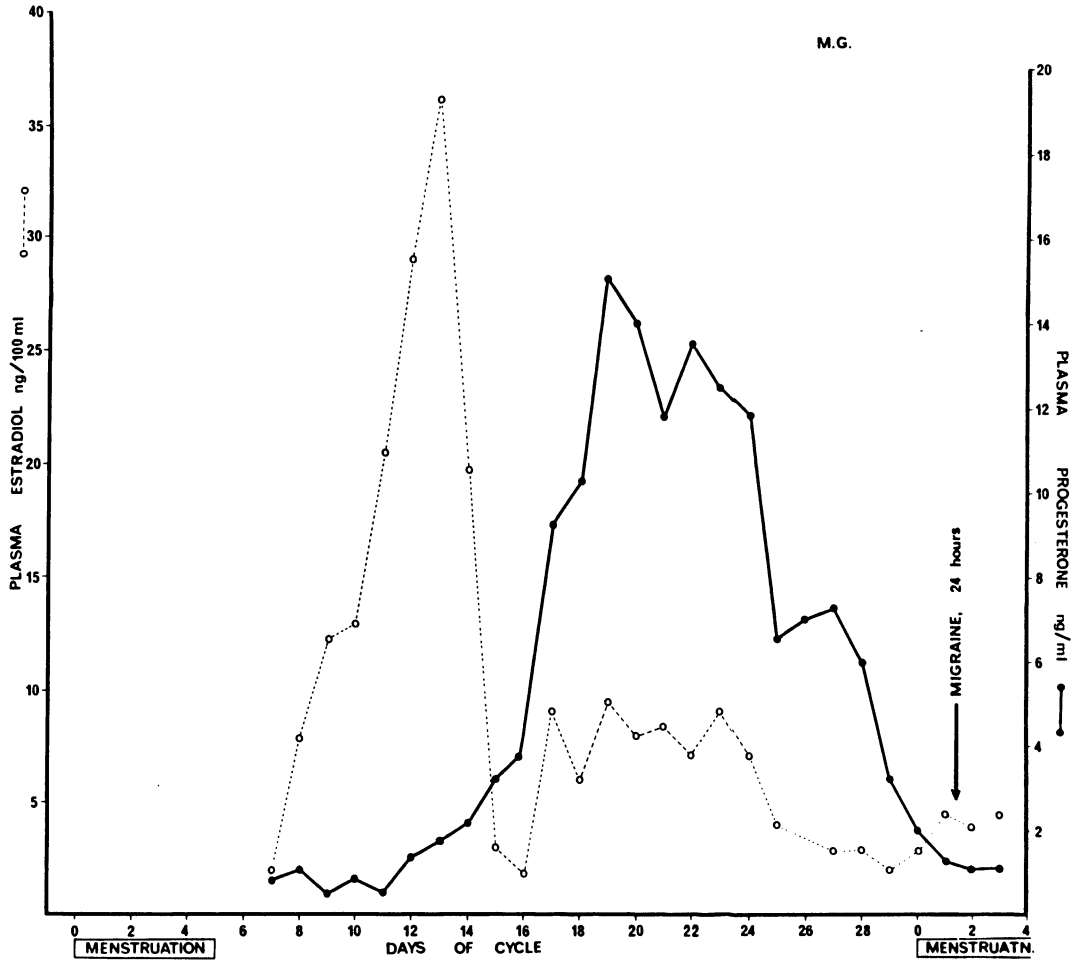
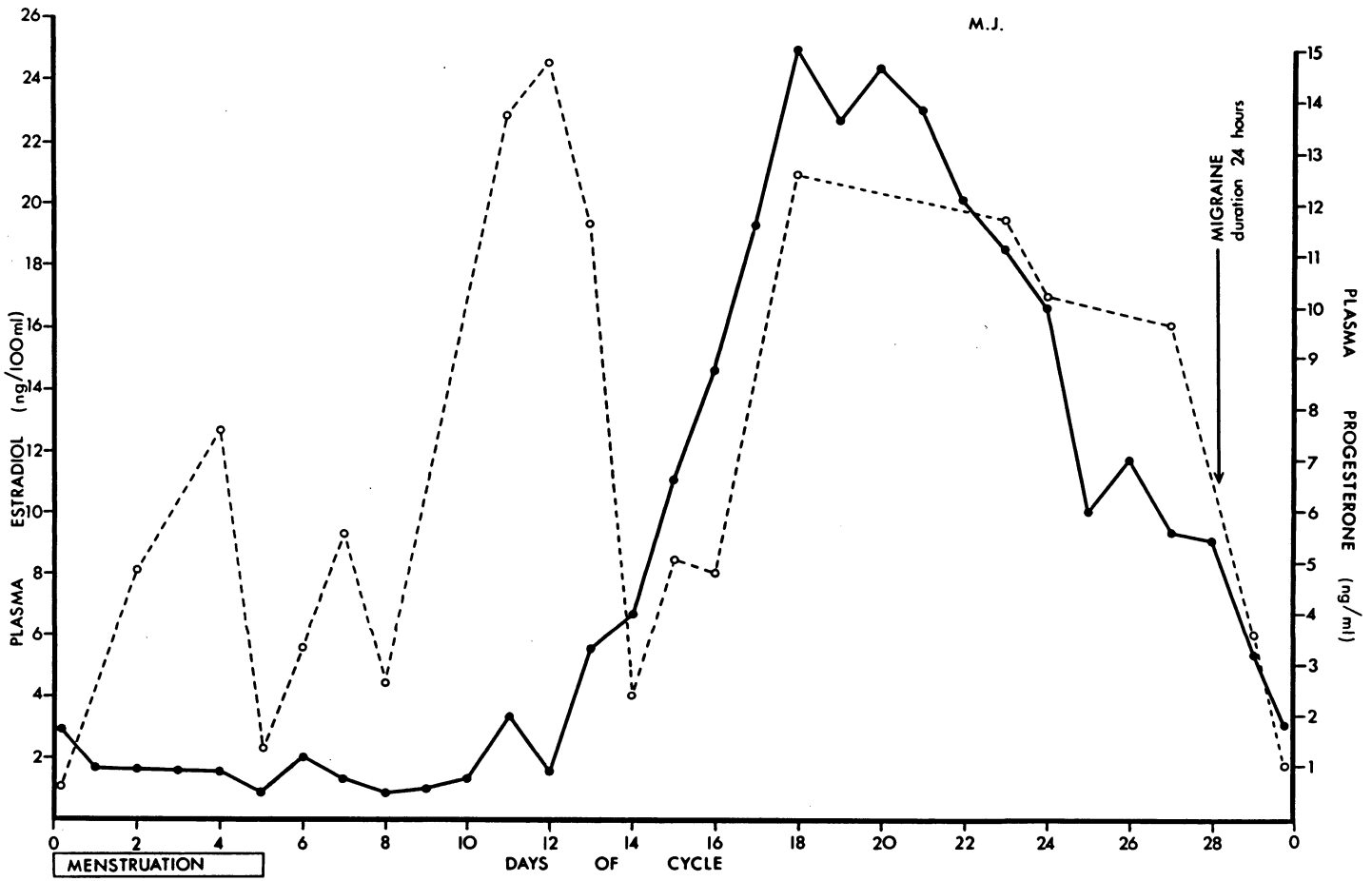

M.G.
PLASMA ESTRADIOL ng/100 ml
PLASMA PROGESTERONE ng/ml
MIGRAINE, 24 hours
MENSTRUATION
DAYS OF CYCLE
MENSTRUATN.

Figure 6.2

Figure 6.3

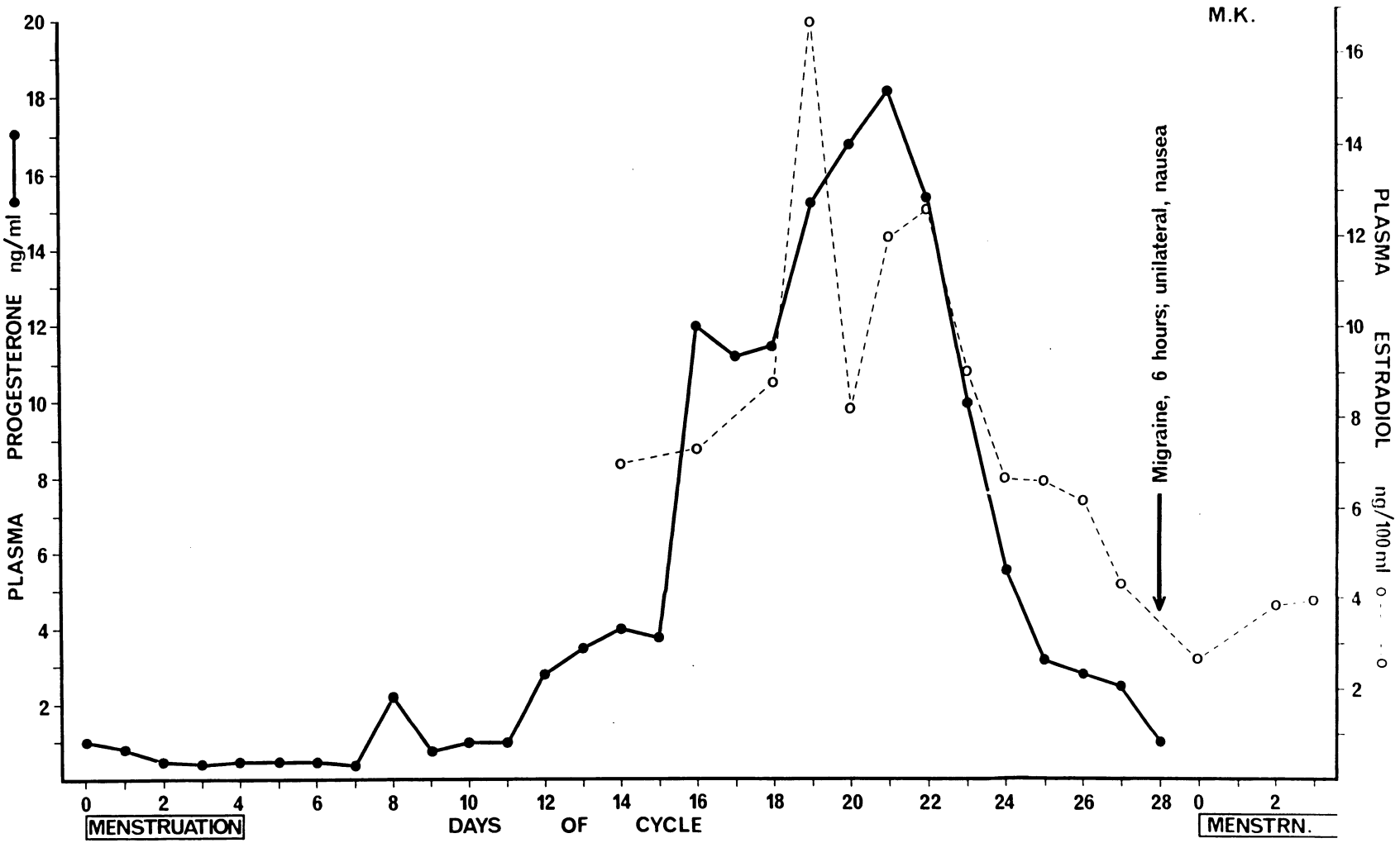

M.J.
PLASMA ESTRADIOL (ng/100ml)
PLASMA PROGESTERONE (ng/ml)
MIGRAINE
duration 24 hours
MENSTRUATION
DAYS OF CYCLE
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
0

Mrs. M.K.

Day of cycle	Progesterone (ng/ml)	Oestradiol (ng/100 ml)
0-7	*	**
8	2.2	**
9	0.8	**
10	1.0	**
11	1.0	**
12	2.8	**
13	3.3	**
14	4.0	7.0
15	3.8	
16	12.0	7.4
17	11.3	
18	11.5	8.7
19	13.2	16.8
20	16.8	8.3
21	18.1	12.0
22	15.3	12.8
23	10.0	9.0
24	5.5	6.7
25	3.1	6.6
26	2.8	6.2
27	2.5	4.3
28	1.0	-

*Values at limit of sensitivity of assay

**Values not obtainable


M.K.
PLASMA PROGESTERONE ng/ml
PLASMA ESTRADIOL ng/100 ml
Migraine, 6 hours; unilateral, nausea
MENSTRUATION
DAYS OF CYCLE
MENSTRN.

CHAPTER 7

THE ROLE OF PROGESTERONE IN MENSTRUAL MIGRAINE

7. THE ROLE OF PROGESTERONE IN MENSTRUAL MIGRAINE

Introduction

Since plasma concentrations of both oestradiol and progesterone had been shown to fall simultaneously during the premenstrual phase, it was necessary to devise a technique of studying the contribution of each hormone separately to the initiation of menstrual migraine.

It was found that, by artificially maintaining the plasma levels of either progesterone or oestradiol through administration of exogenous hormone during the premenstrual phase, it was possible to study the effect of withdrawal of the other hormone. Thus, if progesterone were injected for several days before and during menstruat-ion, the natural decline in oestradiol would continue unaffected, allowing a study to be made of the effect of oestradiol withdrawal alone. Similarly, the administration of oestradiol could be used to study the role of progesterone withdrawal in the aetiology of menstrual migraine. Finally, if administration of the exogenous hormone were discontinued several days after the natural decline in plasma levels of the other hormone, the effect of its decrease in the blood could be studied.

Using this technique, the effect of maintaining a high

level of progesterone during the premenstrual phase, and the effect of progesterone withdrawal were examined.

Subjects

Six regularly-menstruating women, including one from the previous group (M.G.) were studied. All women suffered from regular premenstrual or menstrual migraine, which occurred only during these phases of the cycle. They had all suffered regular monthly headaches for at least 6 cycles immediately preceding the study. The headaches lasted from 8 to 48 hours, and were unilateral in 5 of the 6 subjects. Nausea and photophobia accompanied the headache in all instances. None of the women were taking any hormonal preparation.

Procedure

The latter part of 2 successive cycles was studied in each case. During the first cycle, samples of blood were collected daily and assayed for progesterone and oestradiol as described. Sampling was continued until at least the second day of menstruation. The onset of migraine, its duration, and its relationship to the onset of menstruation were carefully recorded.

In the following, "progesterone-treated" cycle, blood sampling was commenced 3-6 days before the expected date

of menstruation, and daily injections of progesterone in oil ("Proluton", Schering A.G. Berlin) were commenced. These daily injections were continued for several days in order to maintain progesterone at luteal phase levels during the premenstrual and the early part of the menstrual phases. The first 2 women (P.J. and M.G.) were treated with 75 mg daily. When it was found that this dosage had produced higher levels than those found during the previous, control cycle, subsequent patients were treated with a lower dose of 25-50 mg daily, according to the proximity of menstruation. This resulted in the maintenance of progesterone at concentrations normally encountered in the middle of the luteal phase (5-20 ng/ml).

Progesterone injections were discontinued if the subject developed migraine, or if the treatment had been given for several days after the expected onset of menstruation. It was found that a single injection of 25-50 mg maintained the level of progesterone in plasma at luteal phase values for about 36 hours after administration; thereafter, progesterone disappeared rapidly from the blood.

Results

Table 7.1 shows the daily plasma concentrations of progesterone and oestradiol during the latter parts of a normal cycle, and the following progesterone-treated cycle, in each of the 6 women studied. Figures 7.1 to 7.6 have been constructed from these data. Two graphs have been drawn for each patient, which show:

A. Plasma progesterone and oestradiol concentrations during the latter part of a normal cycle (top figure) and during a progesterone-treated cycle (bottom figure)

B. Plasma progesterone concentrations during a normal and a progesterone-treated cycle superimposed.

In both control and progesterone-treated cycles, the onset of migraine has been indicated by a vertical arrow.

It can be seen from the progesterone and oestradiol values during the premenstrual phase of the control cycles, that, in general, the decline in progesterone levels was accompanied by a decline in oestradiol levels. This simultaneous fall in plasma progesterone and oestradiol had been noted previously (Chapters 5 and 6). During the normal control cycle in each of the 6 women studied, migraine occurred during, or at the end of, the phase of withdrawal of progesterone and oestradiol.

Treatment with daily injections of progesterone maintained the plasma progesterone at luteal-phase levels, while the level of oestradiol declined naturally at the end of the cycle. Despite this artificial maintenance of high levels of progesterone, 5 of the 6 women developed migraine at the expected time of the cycle. Four of these women suffered their usual severe headache, while the fifth had a modified, much less severe headache which was shorter in duration than usual (½ hour, instead of the customary 24-48 hours), and which was not accompanied by nausea or visual disturbance. The sixth woman (figure 7.6) did not experience migraine during the progesterone-treated cycle.

Progesterone treatment caused postponement of menstruation in 4 of the 6 women. In these subjects, withdrawal bleeding occurred within 48 hours of the cessation of treatment. This was usually heavier and more prolonged than normal menstrual bleeding. In none of these 4 migrainous women was this progesterone-withdrawal bleeding accompanied by migraine.

In figures 7.1-7.6, hormone values for the progesterone-treated cycle have been plotted around the expected day of onset of menstruation, since treatment with progesterone delayed menstruation in 4 women. The expected onset of bleeding ("day 0") was calculated from the menstrual history, and the fact that all women had regular menstrual cycles.

TABLE 7.1

Days from onset of menstruation	Mrs. V.H. Progesterone ng/ml	Mrs. V.H. Oestradiol ng/100 ml	Mrs. R.V. Progesterone ng/ml	Mrs. R.V. Oestradiol ng/100 ml
CONTROL CYCLE				
-6	9.8	-	-	9.0
-5	13.0	17.0	13.7	7.0
-4	9.8	17.1	8.5	10.3
-3	8.6	21.9	3.7	4.0
-2	5.8	15.3	3.0	2.3
-1	4.2	9.3	0.8	25.0
0	1.8	4.8	0.5	6.3
1	1.0	-	0.5	4.7
2				
PROGESTERONE-TREATED CYCLE				
-6	11.0	25.3	-	10.5
-5	7.8	11.0	5.0	8.3
-4	9.6	11.1	6.4	3.2
-3	8.3	4.0	8.0	2.3
-2	8.8	3.5	4.0	1.0
-1	8.4	3.3	3.5	-
0	8.2	3.7	6.5	2.2
1	5.6	1.7	5.2	1.0
2	3.2	4.6	6.0	-

TABLE 7.1 (contd.)

Days from onset of menses	Mrs. M.G. Progesterone ng/ml	Mrs. M.G. Oestradiol ng/100 ml	Mrs. P.J. Progesterone ng/ml	Mrs. P.J. Oestradiol ng/100ml
CONTROL CYCLE				
-7	-	9.1		
-6	11.0	7.1		
-5	4.4	4.0		
-4	6,4	-		
-3	6.0	3.0	11.0	11.3
-2	4.8	3.1	9.8	19.0
-1	2.5	2.1	3.8	7.7
0	1.0	2.8	3.0	3.7
1	0.5	4.8	1.2	2.2
2			1.3	2.4
PROGESTERONE-TREATED CYCLE				
-6				7.2
-5			13.4	4.4
-4			13.0	1.0
-3	23.4	13.1	14.3	1.0
-2	23.0	3.4	17.0	1.3
-1	19.0	2.1	16.4	1.9
0	22.0	1.6	25.0	1.0
1	15.0	1.0	14.4	1.8
2			19.0	1.0

TABLE 7.1 (contd.)

Days from onset of menses	Mrs. P.R. Progesterone ng/ml	Mrs. P.R. Oestradiol ng/100ml	Mrs. J.B. Progesterone ng/ml	Mrs. J.B. Oestradiol ng/100ml
CONTROL CYCLE				
-6			5.8	7.0
-5			4.2	5.6
-4	9.2	6.5	6.4	6.4
-3	7.6	7.0	3.2	8.6
-2	3.7	5.1	1.6	7.3
-1	3.1	2.1	1.2	2.0
0	1.0	2.2	1.0	1.0
PROGESTERONE-TREATED CYCLE				
-4	4.3	11.3		
-3	4.4	8.6	5.4	10.6
-2	7.8	5.1	6.4	9.9
-1	6.5	5.3	6.2	7.9
0	7.9	2.9	7.8	2.2
1			6.3	0.9
2			5.3	0.8

FIGURES 7.1 (A)&(B) to 7.6(A)&(B)

Plasma levels of progesterone and oestradiol measured daily during the latter part of 2 cycles, in each of 6 women subject to regular menstrual migraine. Two figures have been constructed for each woman:

A. Progesterone and oestradiol levels during a normal cycle (top figure) and during a progesterone-treated cycle (bottom figure).

B. Progesterone levels during a normal cycle, and during a progesterone-treated cycle superimposed, illustrating that in 5 of the 6 women, migraine still occurred at the usual time in the treated cycle, in which progesterone levels were maintained at luteal-phase values.

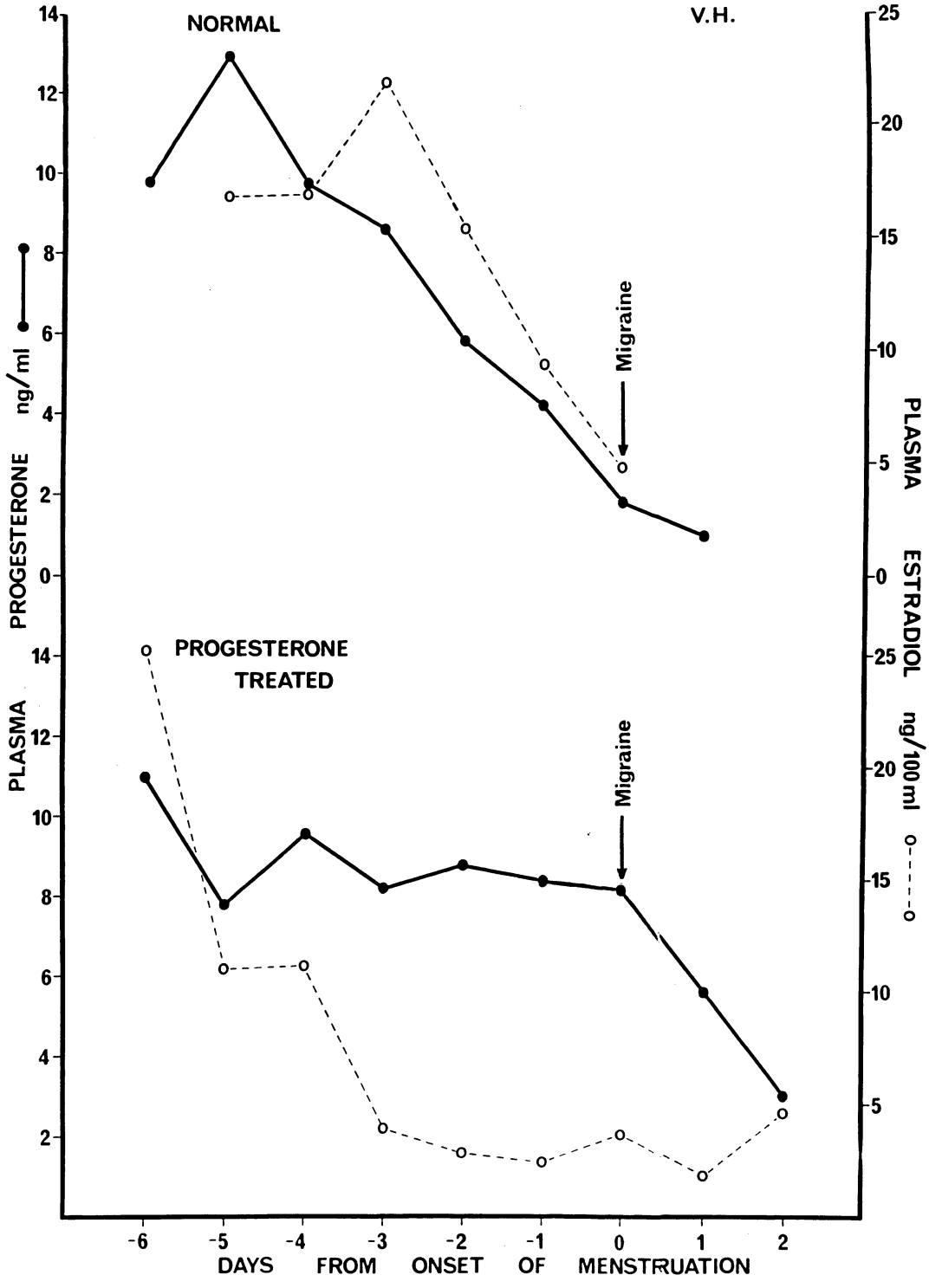

NORMAL
V.H.
Migraine
PROGESTERONE ng/ml
PLASMA ESTRADIOL ng/100 ml
PROGESTERONE TREATED
PLASMA
Migraine
DAYS FROM ONSET OF MENSTRUATION

Figure 7.1(A)

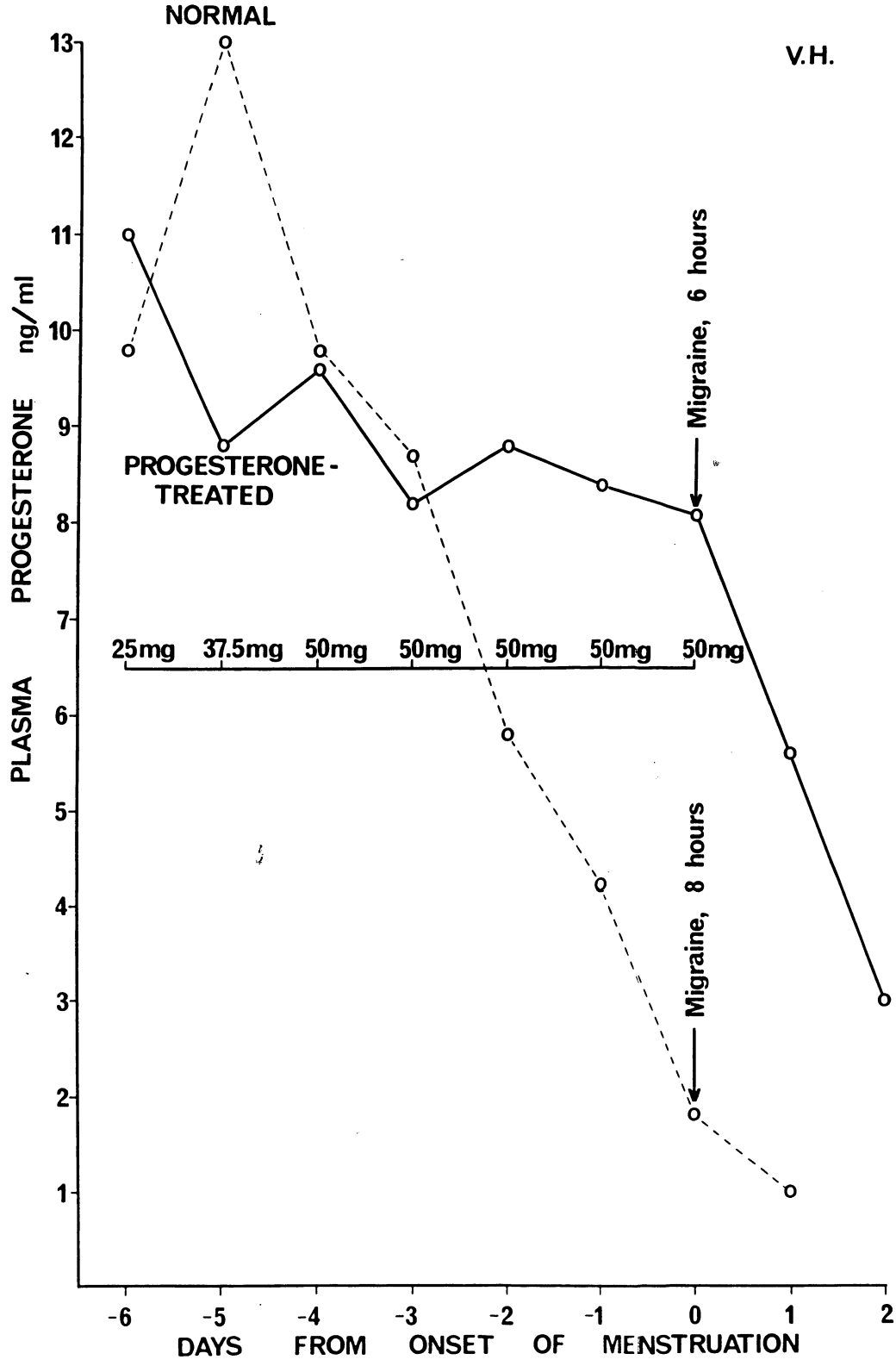

NORMAL
V.H.
PLASMA PROGESTERONE ng/ml
PROGESTERONE-TREATED
25mg 37.5mg 50mg 50mg 50mg 50mg 50mg
Migraine, 6 hours
Migraine, 8 hours
DAYS FROM ONSET OF MENSTRUATION

Figure 7.1(B)

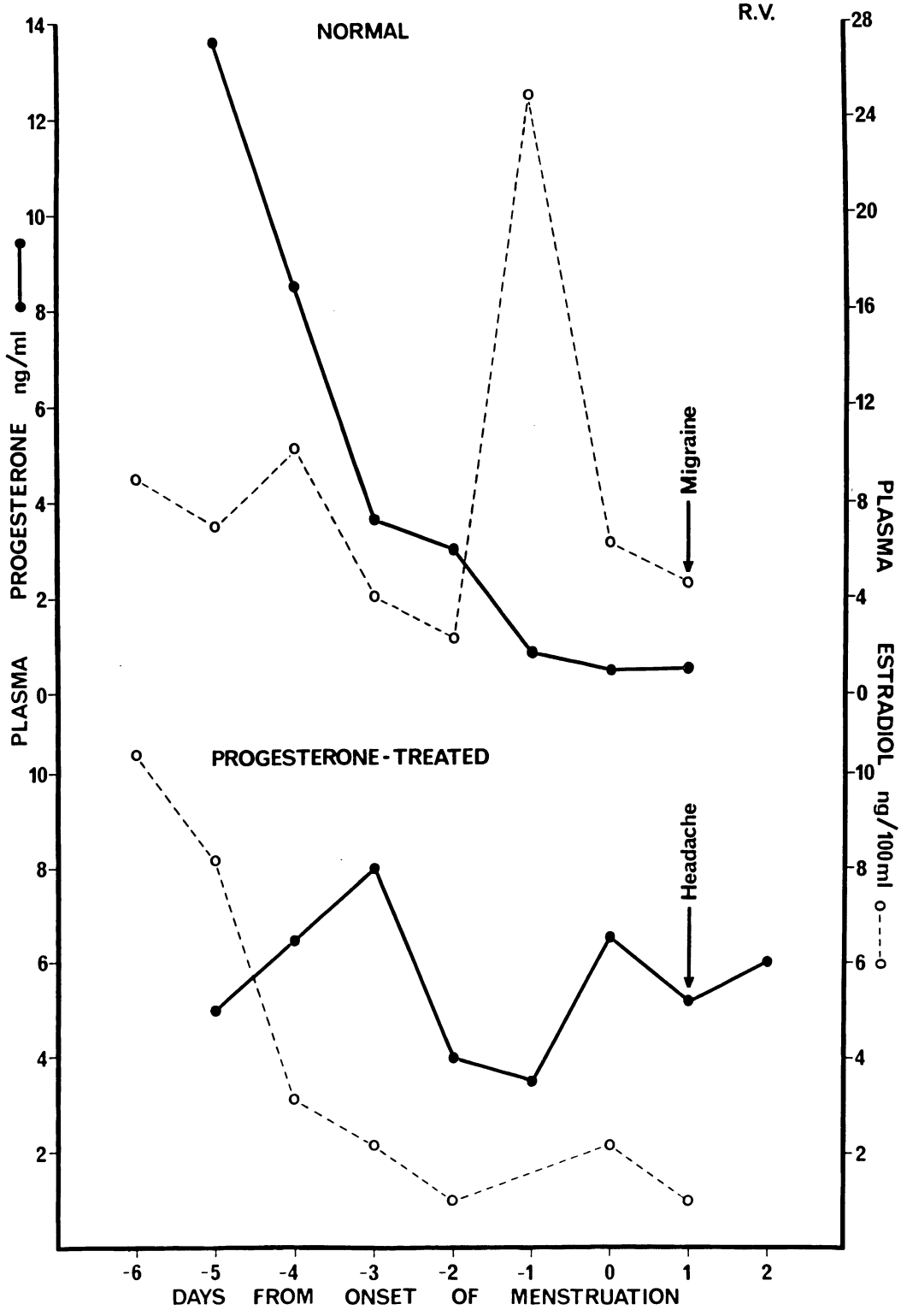

R.V.
NORMAL
PROGESTERONE-TREATED
Migraine
Headache
PLASMA PROGESTERONE ng/ml
PLASMA ESTRADIOL ng/100ml
DAYS FROM ONSET OF MENSTRUATION
-6
-5
-4
-3
-2
-1
0
1
2
14
12
10
8
6
4
2
0
28
24
20
16
12
8
4

Figure 7.2(A)

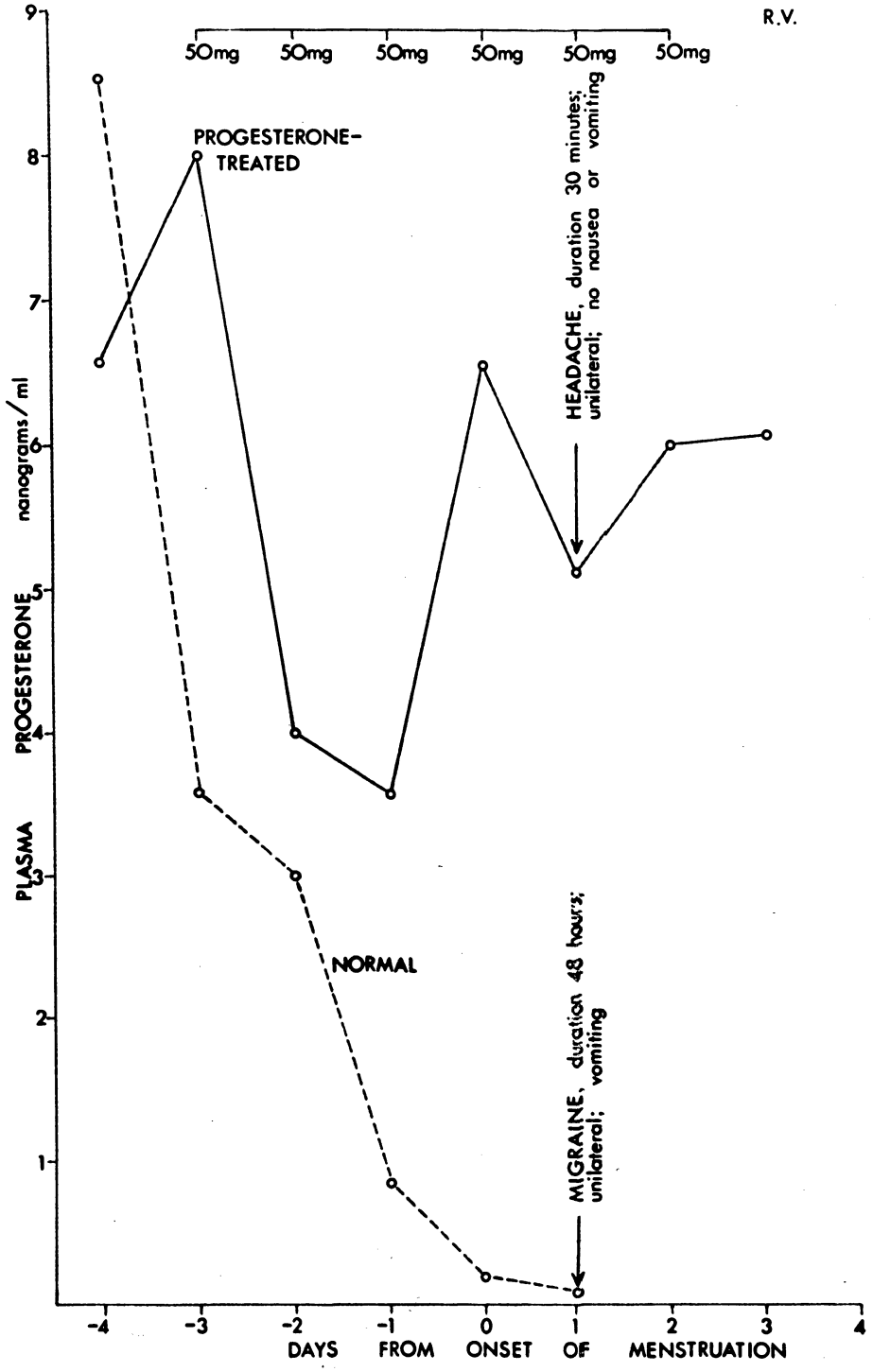

R.V.
50mg
50mg
50mg
50mg
50mg
50mg
PROGESTERONE-
TREATED
HEADACHE, duration 30 minutes;
unilateral; no nausea or vomiting
NORMAL
MIGRAINE, duration 48 hours;
unilateral; vomiting
PLASMA PROGESTERONE nanograms/ml
9
8
7
6
5
4
3
2
1
-4
-3
-2
-1
0
1
2
3
4
DAYS FROM ONSET OF MENSTRUATION

Figure 7.2(b)

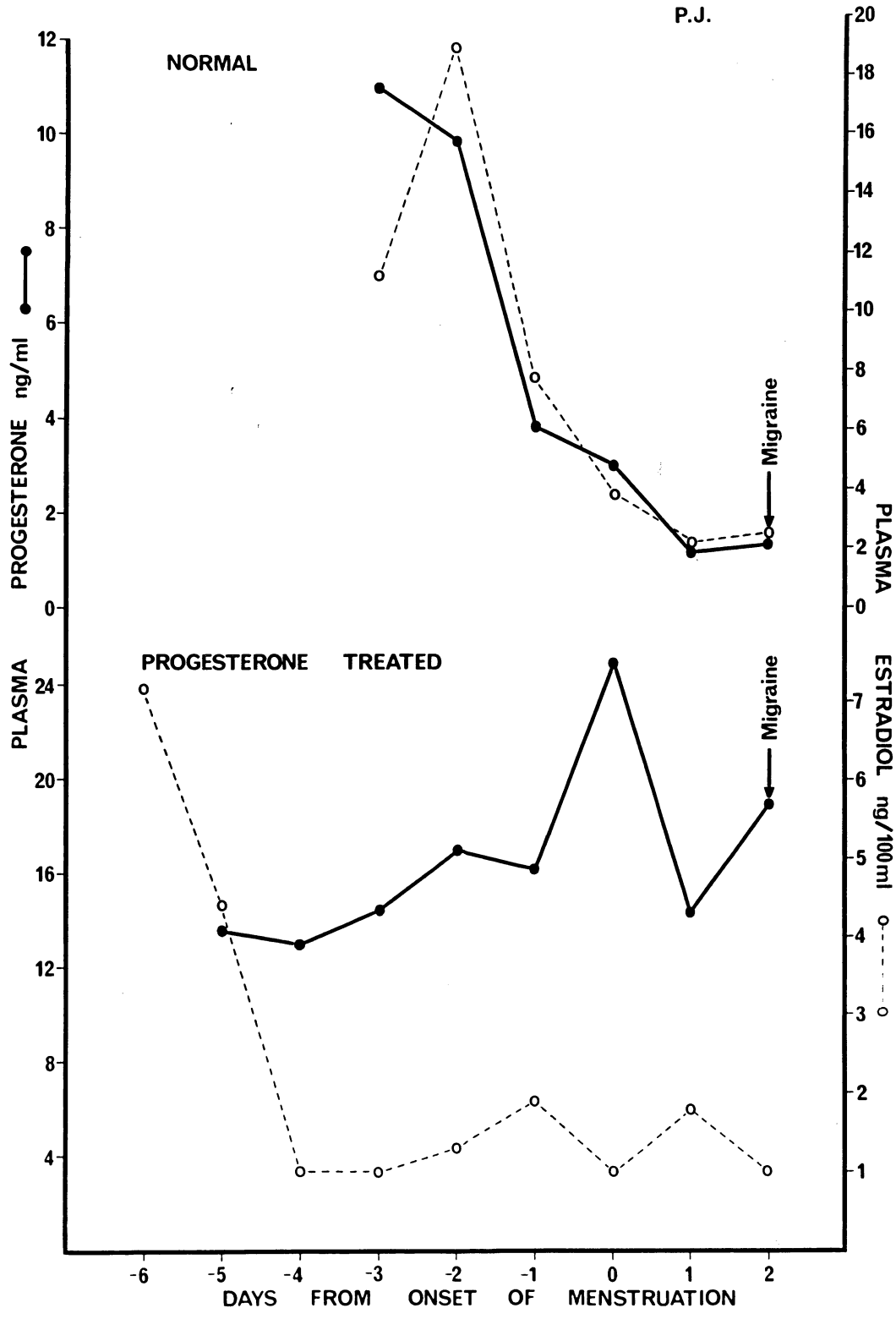

P.J.
NORMAL
PROGESTERONE ng/ml
PLASMA
ESTRADIOL ng/100ml
Migraine
PROGESTERONE TREATED
Migraine
-6 -5 -4 -3 -2 -1 0 1 2
DAYS FROM ONSET OF MENSTRUATION

Figure 7.3(A)

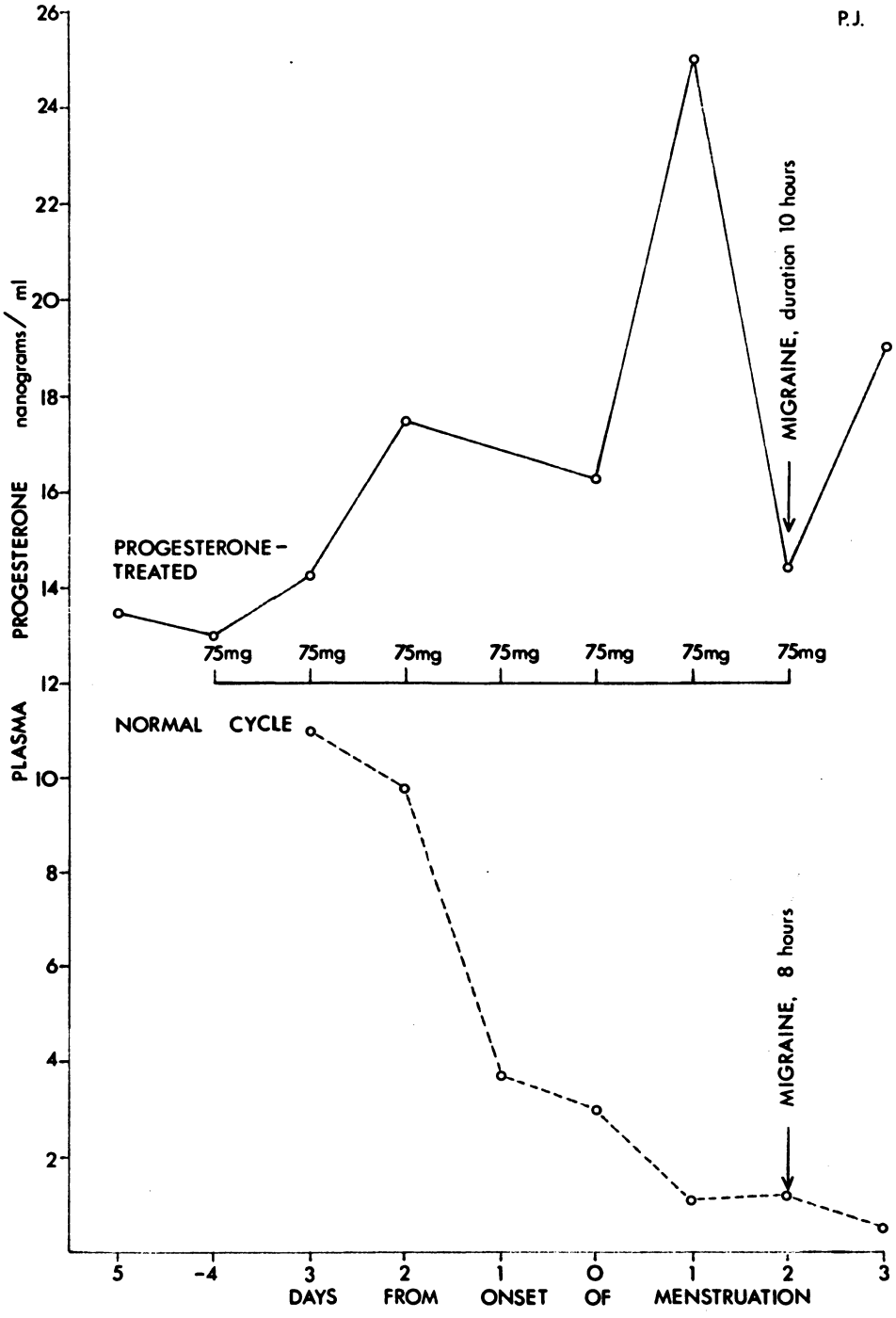

P.J.
PLASMA PROGESTERONE nanograms / ml
26
24
22
20
18
16
14
12
10
8
6
4
2
PROGESTERONE - TREATED
75mg
75mg
75mg
75mg
75mg
75mg
75mg
MIGRAINE, duration 10 hours
NORMAL CYCLE
MIGRAINE, 8 hours
5
-4
3
2
1
O
1
2
3
DAYS FROM ONSET OF MENSTRUATION

Figure 7.3(B)

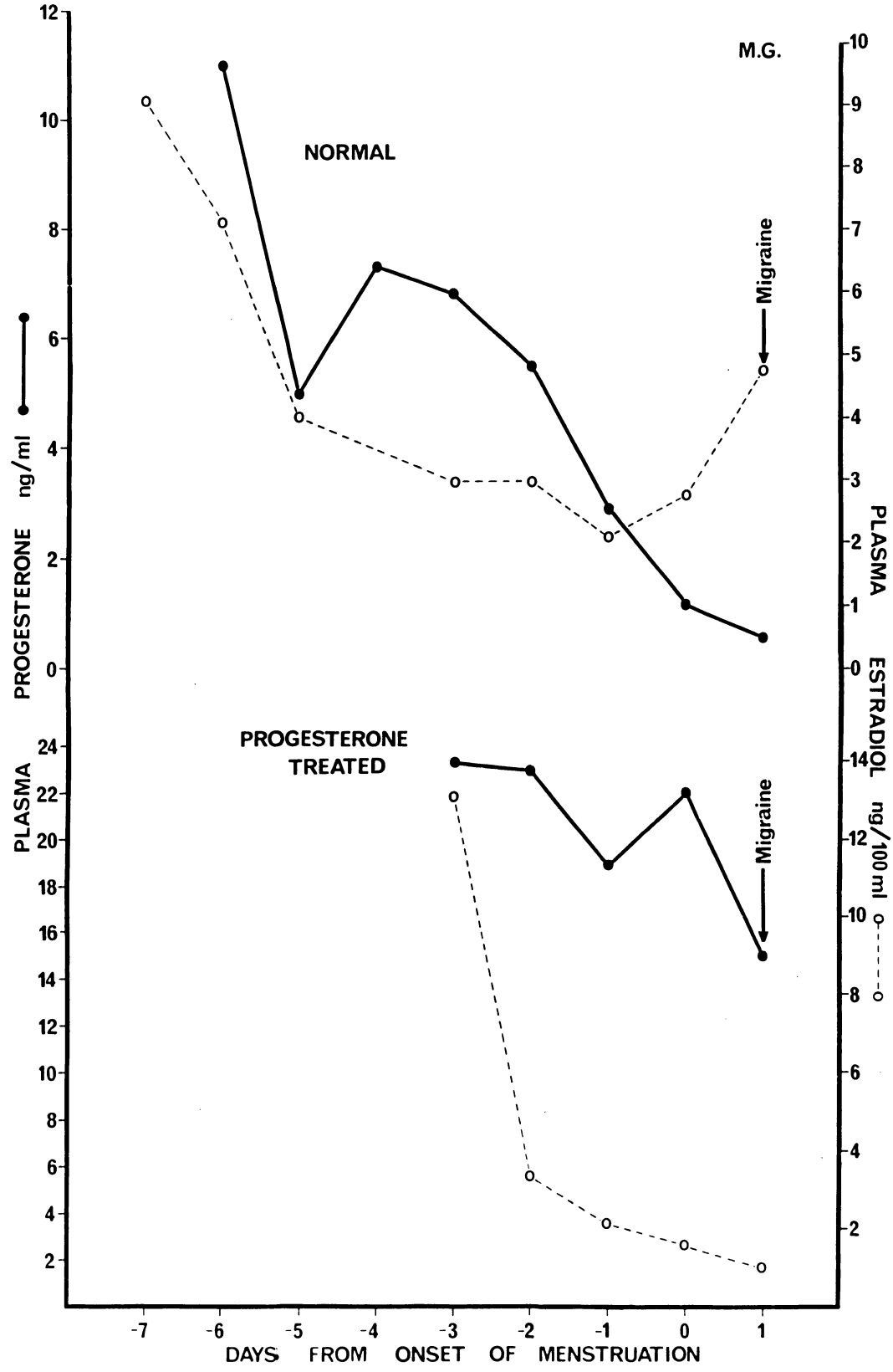

M.G.
NORMAL
Migraine
PROGESTERONE ng/ml
PLASMA ESTRADIOL ng/100 ml
PROGESTERONE TREATED
Migraine
DAYS FROM ONSET OF MENSTRUATION

Figure 7.4(A)

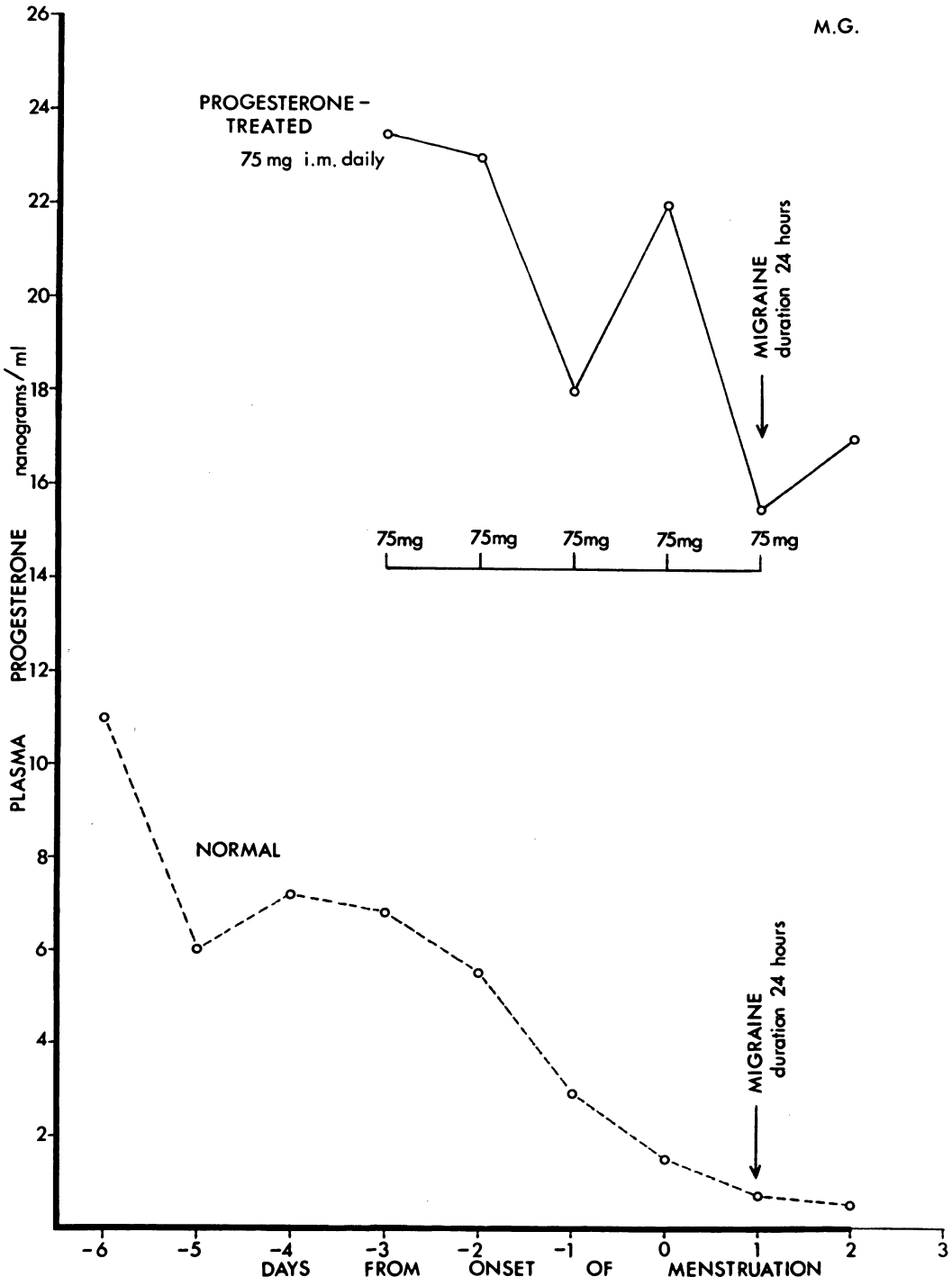

M.G.
PROGESTERONE – TREATED
75 mg i.m. daily
MIGRAINE duration 24 hours
75mg 75mg 75mg 75mg 75mg
NORMAL
MIGRAINE duration 24 hours
PLASMA PROGESTERONE nanograms / ml
26
24
22
20
18
16
14
12
10
8
6
4
2
-6 -5 -4 -3 -2 -1 0 1 2 3
DAYS FROM ONSET OF MENSTRUATION

Figure 7.4(B)

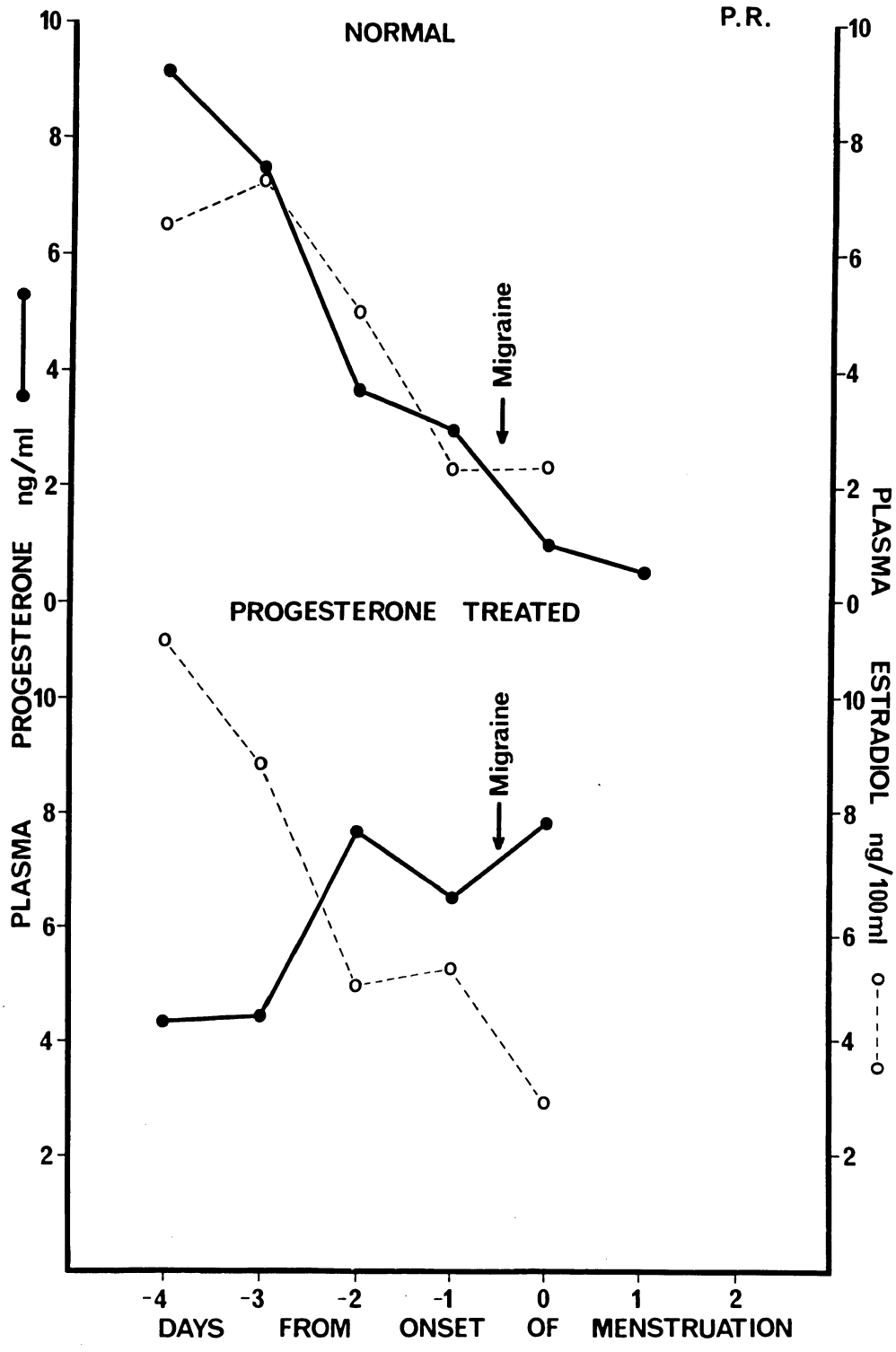

NORMAL
P.R.
Migraine
PROGESTERONE TREATED
Migraine
PLASMA PROGESTERONE ng/ml
PLASMA ESTRADIOL ng/100ml o- - - -o
10
8
6
4
2
0
-4
-3
-2
-1
0
1
2
DAYS FROM ONSET OF MENSTRUATION

Figure 7.5(A)

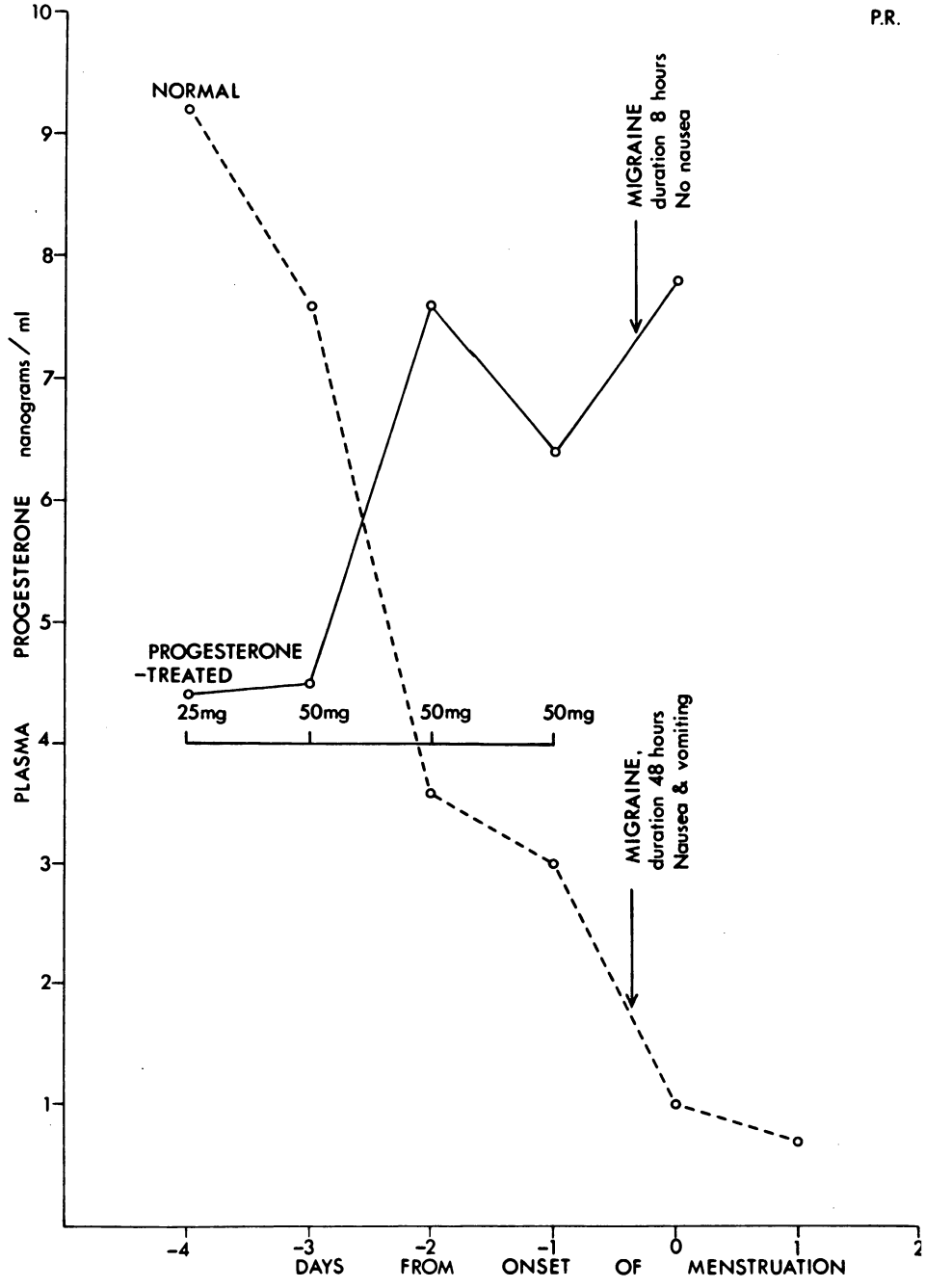

P.R.
NORMAL
MIGRAINE
duration 8 hours
No nausea
PROGESTERONE
-TREATED
25mg
50mg
50mg
50mg
MIGRAINE,
duration 48 hours
Nausea & vomiting
PLASMA PROGESTERONE nanograms / ml
10
9
8
7
6
5
4
3
2
1
-4
-3
-2
-1
0
1
2
DAYS FROM ONSET OF MENSTRUATION

Figure 7.5(B)

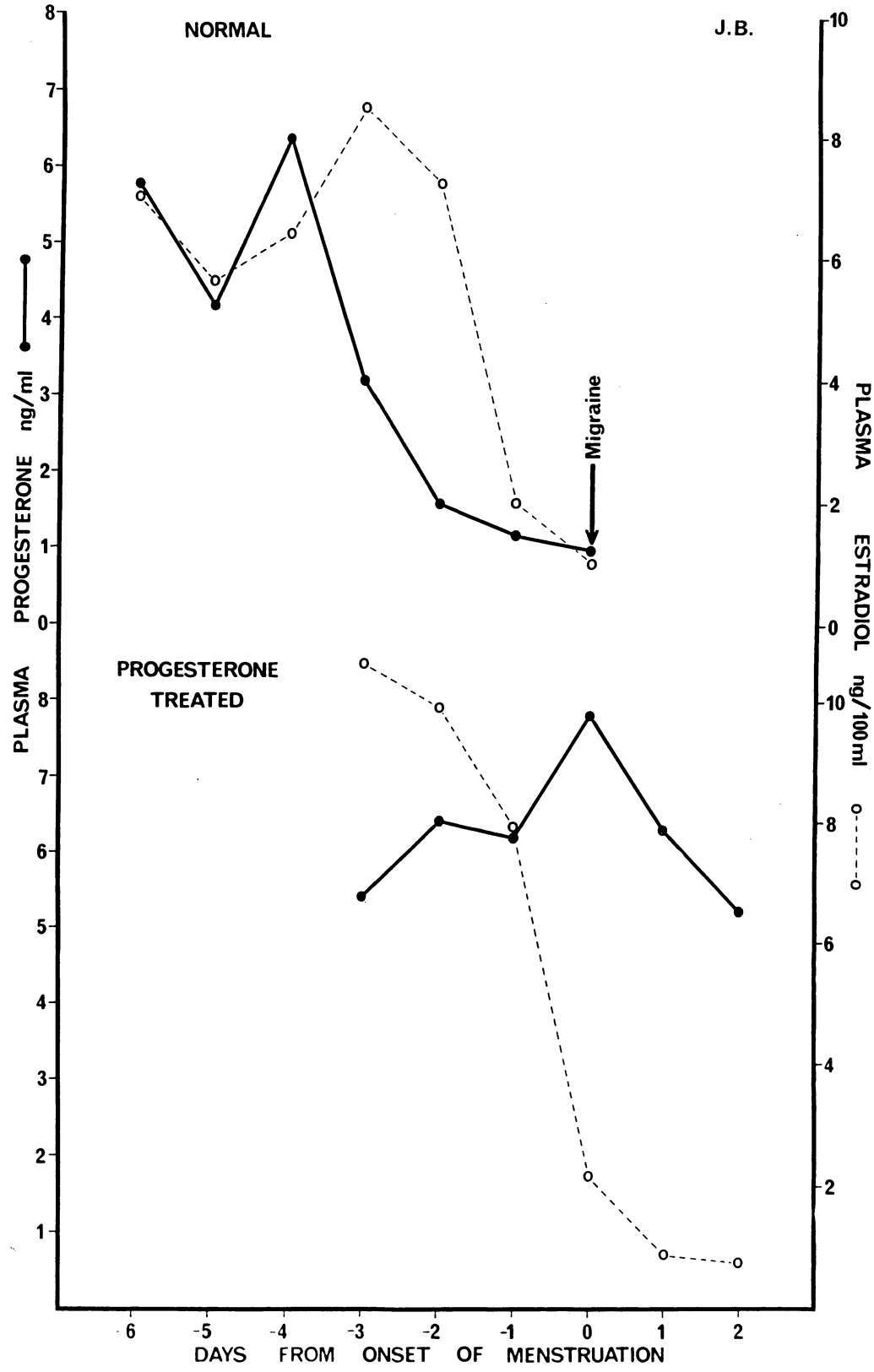

NORMAL
J.B.
PLASMA PROGESTERONE ng/ml
PLASMA ESTRADIOL ng/100 ml
Migraine
PROGESTERONE TREATED
DAYS FROM ONSET OF MENSTRUATION
-6
-5
-4
-3
-2
-1
0
1
2

Figure 7.6(A)

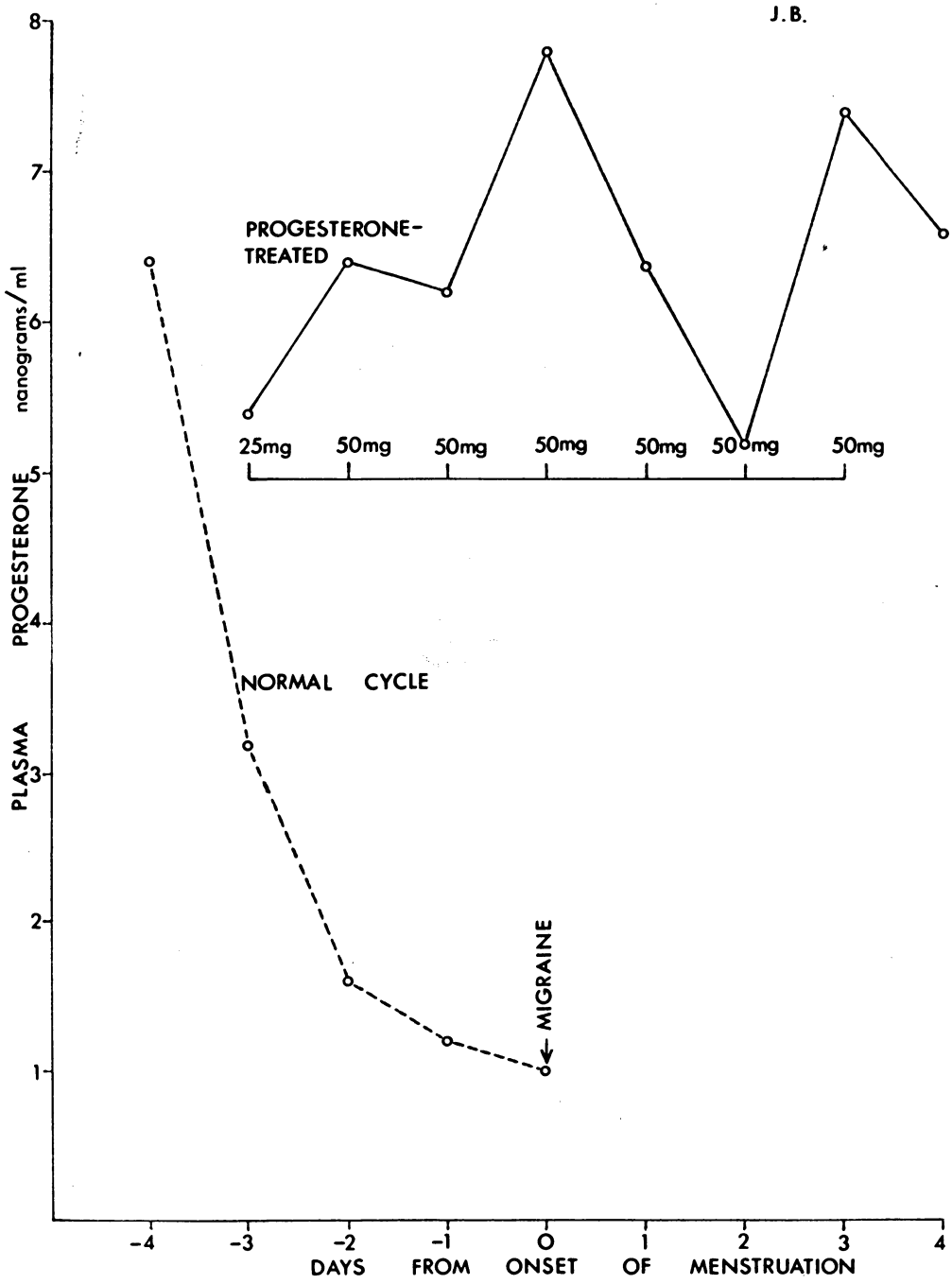

J.B.
PROGESTERONE-
TREATED
25mg
50mg
50mg
50mg
50mg
50mg
50mg
NORMAL CYCLE
MIGRAINE
PLASMA PROGESTERONE nanograms/ml
8
7
6
5
4
3
2
1
-4
-3
-2
-1
0
1
2
3
4
DAYS FROM ONSET OF MENSTRUATION

Figure 7.6(B).

Discussion

The pattern of oestradiol and progesterone levels obtained during the control cycles confirms that menstrual migraine tends to occur during, or at the termination of the phase of falling plasma levels of these hormones.

From a clinical study of women experiencing menstrual migraine while taking synthetic oestrogen-progestogen combinations for contraception, Whitty et al. (1966) suggested that menstrual migraine may be precipitated by the withdrawal of exogenous progestogen. In the current study, plasma levels of the natural hormones, progesterone and oestradiol, were measured, since suitable micromethods for the measurement of synthetic progestogenic and oestrogenic hormones were not available.

In the absence of data concerning the blood levels of these synthetic hormones in women subject to menstrual migraine, it would be impossible to deny that withdrawal of synthetic progestogen were the precipitating cause of migraine. However, in view of the similarity of the physiological actions of the natural and synthetic hormones, it would appear unlikely that withdrawal of either natural or synthetic progesterone were the cause of menstrual migraine since, in this study, treatment with progesterone aimed at

preventing the usual fall in progesterone during the premenstrual phase failed to prevent migraine in 5 of the 6 women studied.

The ineffectiveness of progesterone in preventing migraine in this study contrasts to earlier reports, including those of Gray (1941); Singh, Singh and Singh, (1947); Steinkamm, (1951); Blumenthal and Fuchs, (1951); Kinnunen and Mustakallio, (1952); Greene and Dalton (1953); Keil, (1953); Schneider, (1955); Schwenzer (1956); and Sack and Handrick, (1956). These earlier workers in general used much smaller doses of progesterone injected at longer intervals. Such schedules of treatment would not be expected to cause any significant elevation in the plasma level of progesterone.

There is also a notable lack of criteria for defining "migraine" in these various series- some authors appear to use the terms "migraine" and "headache" as synonymous. Although the author's series is small, it is the first study in which plasma concentrations of progesterone have been measured, and in which progesterone has been administered in a sufficient dose to maintain progesterone levels at luteal phase values. The failure of progesterone withdrawal to precipitate migraine in any of the women must cast serious doubt on the hypothesis that progestogen withdrawal is the precipitating cause of menstrual migraine.

It is noteworthy that in 3 of the 4 women whose menstruation was delayed by the progesterone treatment, migraine still occurred at the expected time. This illustrates that the processes of uterine bleeding and menstrual migraine may occur independently. Furthermore, in none of these 4 women was the cessation of progesterone treatment and the subsequent withdrawal bleeding associated with migraine.

Since these women were receiving a potent hormone preparation, it was necessary for both patient and doctor to be aware of the nature of the treatment. Thus it was not possible to carry out a "double blind" type of investig--ation. The failure of one of the women to develop migraine, and the attenuation of the migraine attack in a second woman during the progesterone-treated cycle may have been due, therefore, to placebo effect.

SUMMARY

The following points emerge from this study:

1. Naturally-occurring menstrual migraine usually begins during, or just after the phase of simultaneous withdrawal of progesterone and oestradiol.

2. The patterns of plasma oestradiol and progesterone concentrations during the menstrual cycle in women subject to menstrual migraine do not differ significantly from those of normal, non-migrainous women.

3. Under experimental conditions in which a high plasma level of progesterone is maintained, menstrual migraine may occur in the absence of uterine bleeding.

4. The administration of progesterone during the premenstrual phase appears to be ineffective in preventing menstrual migraine.

5. Withdrawal of progesterone alone does not appear to be the precipitating cause of menstrual migraine.

CHAPTER 8 - THE ROLE OF OESTRADIOL IN MENSTRUAL MIGRAINE

Introduction

In the previous chapter, an account has been given of the effect of preventing the normal premenstrual decline in plasma progesterone on menstrual migraine. Using a similar experimental model, the effect of artific-ially maintaining a high level of oestradiol during the premenstrual phase was investigated.

Subjects

This study included 5 of the 6 women studied during "control" and "progesterone-treated" cycles. A sixth woman, whose plasma oestradiol and progesterone levels had been studied daily throughout an entire (untreated) cycle, was added to this group. All 6 women had experienced regular, predictable menstrual or premenstrual migraine, for at least 6 successive cycles immediately prior to this investigation. The characteristics of these headaches have been described previously.

In addition to these 6 regularly-menstruating women, 2 other migrainous women were studied. One of these had experienced regular menstrual migraine for many years, but had developed menopausal amenorrhoea, with cessation of migraine, 3 months before the investigation. The other woman was subject to recurrent failure of ovulation. In her

case, 3 or 4 successive cycles, each accompanied by typical menstrual migraine, would be followed by an interval of several months of amenorrhoea. In all other respects, the migrainous headaches of these latter 2 subjects resembled those of the 6 regularly menstruating women.

Procedure

The latter part of two cycles was studied. During the first, or "control" cycle, no treatment was given, while during the second, oestradiol was administered. To maintain high plasma levels of oestradiol for several days, one intramuscular injection of oestradiol in sesame oil ("Progynova", Schering A.G., Berlin) was administered 3-10 days before the expected onset of menstruation. At first, one single injection of 20 mg was used; later, it was found that this produced a very high plasma oestradiol concentration (above 140 ng/100 ml), so that the dose was reduced to 10 mg, given as a single injection, or as two injections of 5 mg each, separated by several days. The aim of this oestradiol treatment was to maintain a high level of the hormone during the premenstrual and early menstrual phases, and thereby to prevent the usual physiological decline in oestradiol levels which occurs at this time.

During the latter part of the control cycle, and before and during oestradiol treatment in the subsequent cycle,

blood samples of 10 ml were obtained daily for progesterone and oestradiol assays. Progesterone determinations were performed in duplicate using 0.5 ml aliquots of plasma, while oestradiol assays were performed using single 1-4 ml samples of plasma. As the injection of oestradiol frequently resulted in high plasma levels for the first few days, plasma samples were suitably diluted with double-distilled water in order that the determination would fall on the linear portion of the standard curve.

In the case of the 2 migrainous women who were not menstruating, one blood sample was obtained before administering oestradiol. Daily blood sampling was then continued until the onset of migraine.

The women were informed that they were receiving hormone treatment, and that some menstrual upset might be expected. They were not informed of the effect that this treatment might have upon their migraine.

Results

The plasma oestradiol and progesterone values during the control cycles have been shown in Chapter 7. The values obtained during the oestradiol-treated cycles are shown in the following tables. From these data, graphs have been constructed showing plasma oestradiol concentrations during the control cycle, and during the oestradiol-treated cycle, in each of the 6 regularly-menstruating women. These graphs are shown in figures 8.1 to 8.6.

It is apparent that this form of treatment caused a sustained elevation of plasma oestradiol, and therefore succeeded in preventing the normal physiological drop in plasma oestradiol which would otherwise have occurred in the premenstrual phase. It did not, however, affect the normal premenstrual decline in plasma levels of progesterone (figures 8.7 to 8.11).

The injection of oestradiol valerate caused a marked and rapid increase in plasma oestradiol concentrations in all subjects. There was considerable individual variation in peak concentrations of oestradiol attained. Thus, in one patient (figure 8.6), a single injection of oestradiol valerate 10 mg produced a plasma oestradiol concentration of above 200 ng/100 ml, while in another (figure 8.5), a similar dose raised the plasma level of oestradiol to

only 112.0 ng/100 ml. In all cases, the injection of oestradiol was followed by a "plateau" of sustained high levels, which lasted for some days before a decline to pre-injection values became apparent. The sustained effect of a single injection of oestradiol valerate was attributed to its slow release from its oily depot.

The effect of this oestradiol treatment on menstrual migraine was consistent. In all 6 of the regularly-menstruating subjects, migraine was delayed by 3 to 9 days. Five women experienced their usual migraine attack, with its customary severity and associated symptoms, such as blurred vision, nausea, and vomiting. The remaining woman (Mrs. J.B. figure 8.3) experienced a bilateral headache instead of the usual unilateral migraine. This modified headache lasted for 48 hours, and was not accompanied by nausea or vomiting. The same subject had failed to develop migraine during a previous, progesterone-treated cycle.

It was found that migraine began after the oestradiol level had fallen to below 20 ng/100 ml in 5 women. In the case of the sixth subject, an attempt was made to maintain plasma oestradiol levels for a longer period by giving two injections of oestradiol valerate, separated by 6 days. In this instance it was found that, in the interval between injections, the plasma oestradiol level had fallen to

as low as 12.1 ng/100 ml on the sixth day, and, although a second injection was then given, the migraine attack was not further postponed, and commenced while the plasma level of oestradiol was, in fact, rising.

This oestradiol treatment had no effect on the onset of menstruation, which took place at the expected time in all subjects. This menstrual bleeding was shown to be the result of progesterone withdrawal. Figures 8.7 to 8.11 show the plasma levels of oestradiol and progesterone during the oestradiol-treated cycle. It can be seen that the decrease in progesterone levels occurred during the premenstrual phase, despite the artificial maintenance of high plasma concentrations of oestradiol.

In the case of the two women who were not menstruating regularly, the injection of oestradiol was followed by a similar pattern of elevation then decline of plasma levels of the hormone. Plasma progesterone determinations before and during oestradiol treatment in these women revealed consistently low values (less than 2 ng/ml), consistent with absence of ovulation. One woman (Mrs. B.J.W., fig. 8.12) developed oestrogen withdrawal bleeding 11 days after the injection. This was preceded by a typical attack of migraine the day before menstruation. The other woman (Mrs. M.C., figure 8.13) who had become menopausal, did not show any

uterine bleeding in response to oestradiol withdrawal, but suffered her typical episode of migraine 9 days after the injection of oestradiol.

Oestradiol treatment of the 6 regularly menstruating women invariably caused some menstrual disturbance, mainly increased blood loss or prolongation of the bleeding In 3 of these woman, the migraine attack was so postponed that it began some days after menstruation had ceased.

TABLES 8.1 and 8.2

Plasma concentrations of oestradiol and progesterone measured daily during the oestradiol-treated cycles of 6 regularly-menstruating women subject to menstrual migraine.

Table 8.1

Day of cycle	Progesterone ng/ml	Oestradiol ng/100ml	Progesterone ng/ml	Oestradiol ng/100ml
	MRS. P.J.		MRS. P.R.	
-5			6.4	14.6
-4			3.2	112.0
-3	6.5	24.0	3.6	86.7
-2	1.0	82.0	1.0	32.2
-1		38.0	0.6	18.6
0		131.0	-	-
1	Values at limit of sensitivity	200	0.8	12.1
2		-	0.5	138.0
3		127.5		
4		54.3		
7		14.5		
8		8.1		

Table 8.2

Day of cycle	Progesterone ng/ml	Oestradiol ng/100ml	Progesterone ng/ml	Oestradiol ng/100ml
	MRS. J.B.		MRS. R.V.	
-7			3.4	77.0
-6			3.0	59.5
-5			3.6	28.8
-4	19.4	12.2	4.4	20.4
-3	3.0	129.0	4.0	18.2
-2	6.0	87.0	4.2	12.9
-1	1.0	143.5	0.8	8.6
0	1.2	74.8	0.8	3.1
1	1.6	58.7		
2	0.8	74.4		
3	0.5	32.4		
4	0.5	7.3		
	MRS. V.H.		MRS. M.J.	
-4	8.4	18.3		
-3	5.2	87.0		6.4
-2	1.5	113.2		200
-1	0.6	60.2		126.0
0	0.5	55.5		200
1	1.0	42.8		-
2	0.8	28.3		25.0
3	0.9	25.6		33.5
4	0.6	13.9		-
5				12.4
8				3.0

Values at limit of sensitivity

FIGURES 8.1 TO 8.6

Plasma levels of oestradiol measured daily during the latter part of two menstrual cycles, one "control" and one "oestradiol-treated" cycle, in each of 6 regularly menstruating women subject to regular premenstrual or menstrual migraine.

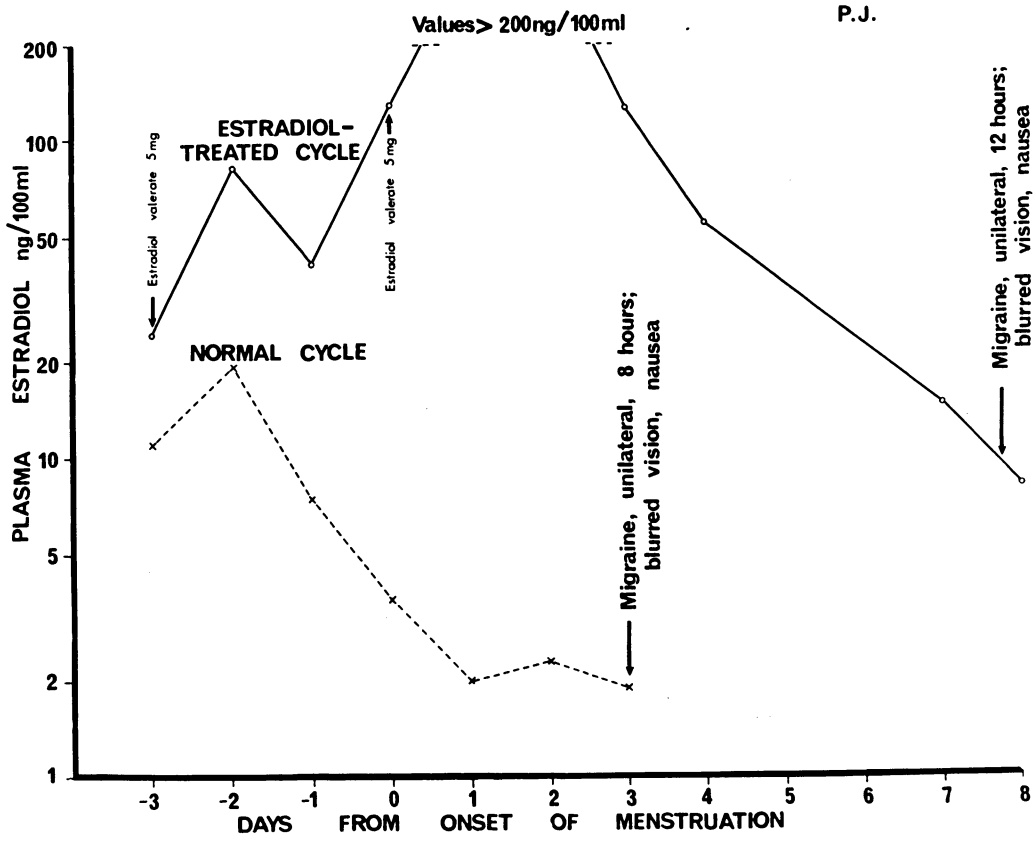

P.J.
Values> 200ng/100ml
PLASMA ESTRADIOL ng/100ml
200
100
50
20
10
5
2
1
ESTRADIOL-
TREATED CYCLE
Estradiol valerate 5mg
Estradiol valerate 5mg
NORMAL CYCLE
Migraine, unilateral, 8 hours;
blurred vision, nausea
Migraine, unilateral, 12 hours;
blurred vision, nausea
-3
-2
-1
0
1
2
3
4
5
6
7
8
DAYS FROM ONSET OF MENSTRUATION

Figure 8.1

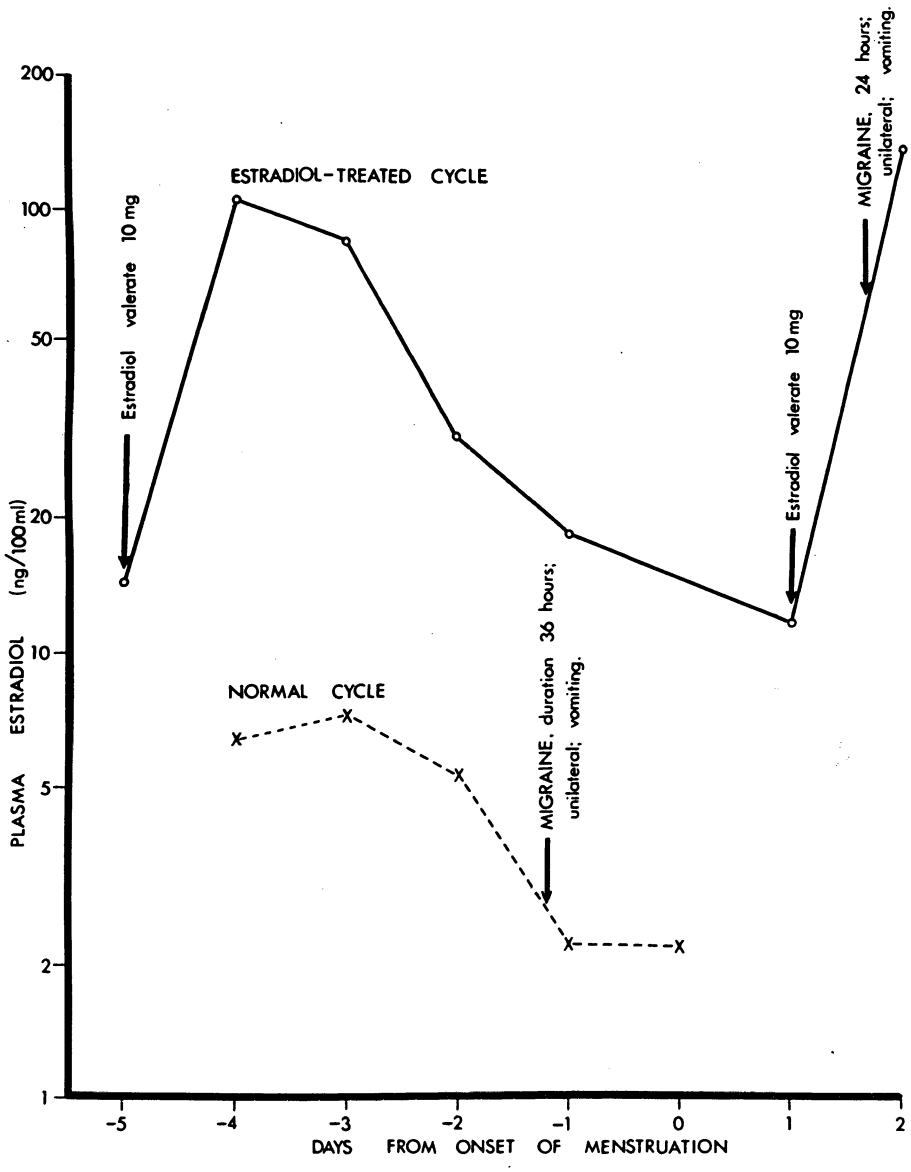

P.R.
ESTRADIOL-TREATED CYCLE
Estradiol valerate 10 mg
Estradiol valerate 10 mg
MIGRAINE, 24 hours; unilateral; vomiting.
NORMAL CYCLE
MIGRAINE, duration 36 hours; unilateral; vomiting.
PLASMA ESTRADIOL (ng/100ml)
200
100
50
20
10
5
2
1
-5
-4
-3
-2
-1
0
1
2
DAYS FROM ONSET OF MENSTRUATION

Figure 8.2

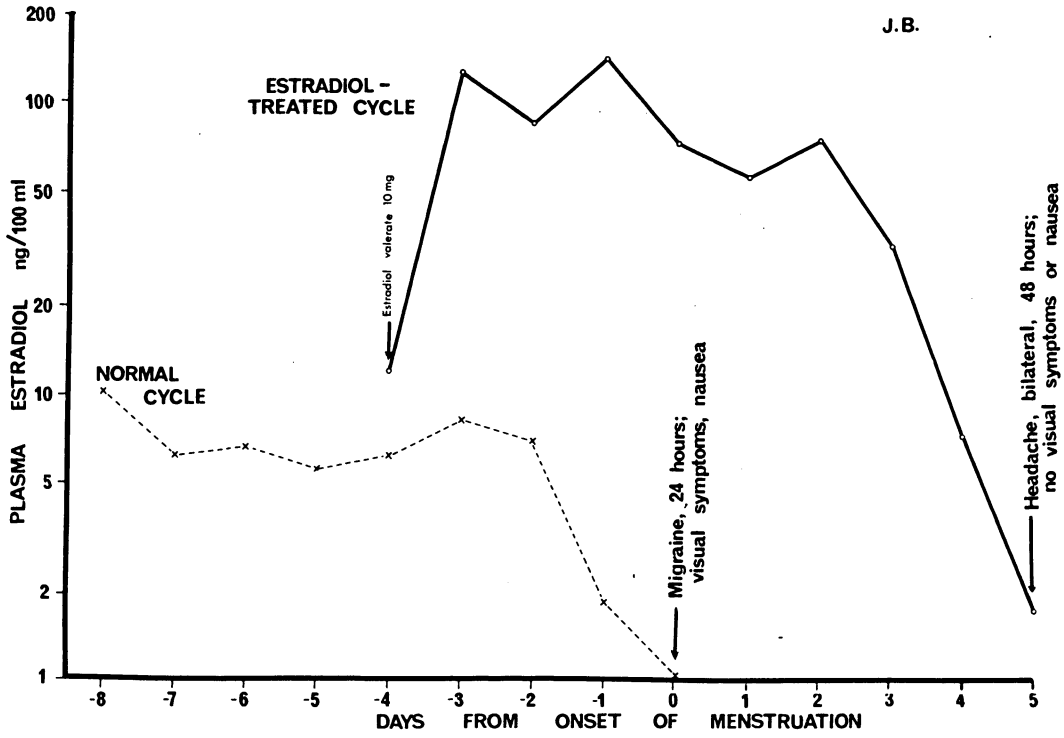

J.B.
ESTRADIOL - TREATED CYCLE
Estradiol valerate 10 mg
NORMAL CYCLE
Migraine, 24 hours; visual symptoms, nausea
Headache, bilateral, 48 hours; no visual symptoms or nausea
PLASMA ESTRADIOL ng/100 ml
200
100
50
20
10
5
2
1
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
DAYS FROM ONSET OF MENSTRUATION

Figure 8.3

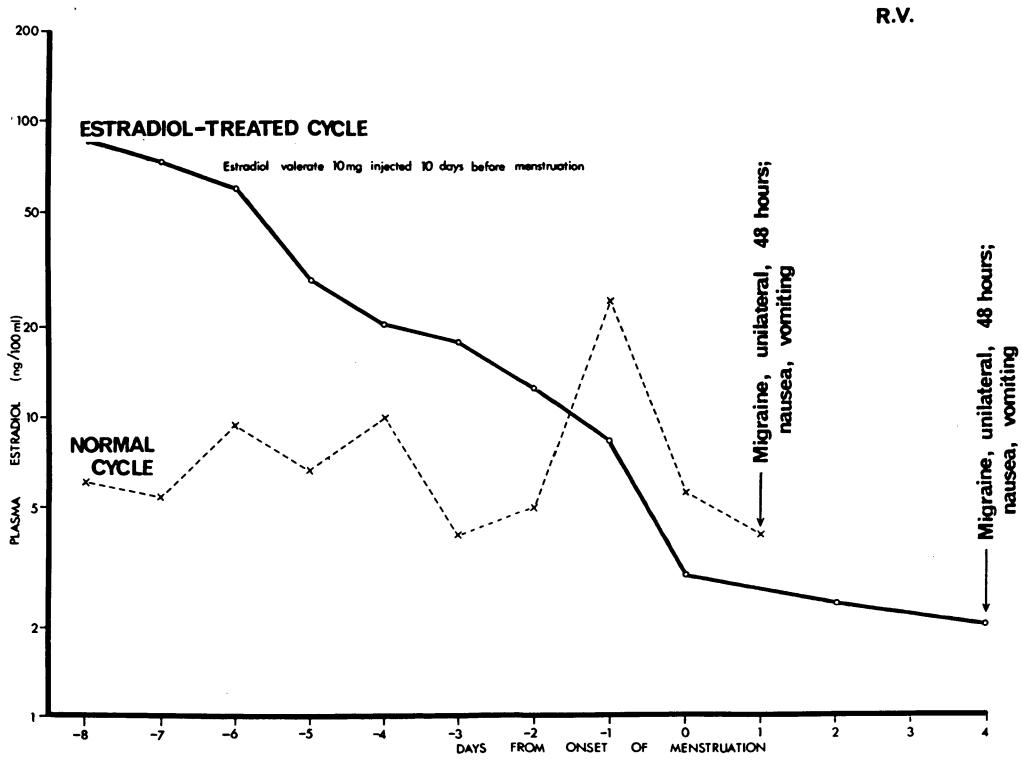

R.V.
ESTRADIOL-TREATED CYCLE
Estradiol valerate 10 mg injected 10 days before menstruation
NORMAL CYCLE
PLASMA ESTRADIOL (ng/100 ml)
200
100
50
20
10
5
2
1
Migraine, unilateral, 48 hours; nausea, vomiting
Migraine, unilateral, 48 hours; nausea, vomiting
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
DAYS FROM ONSET OF MENSTRUATION

Figure 8.4

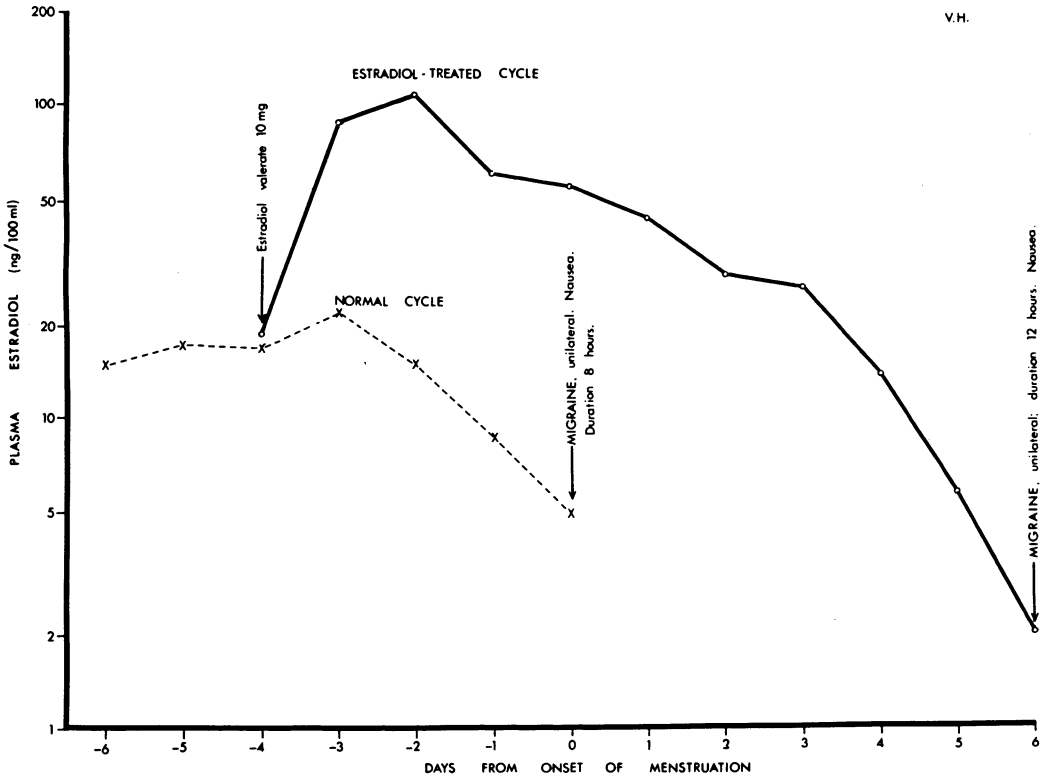

V.H.
ESTRADIOL - TREATED CYCLE
Estradiol valerate 10 mg
NORMAL CYCLE
MIGRAINE, unilateral. Nausea. Duration 8 hours.
MIGRAINE, unilateral; duration 12 hours. Nausea.
PLASMA ESTRADIOL (ng/100 ml)
200
100
50
20
10
5
2
1
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
DAYS FROM ONSET OF MENSTRUATION

Figure 8.5

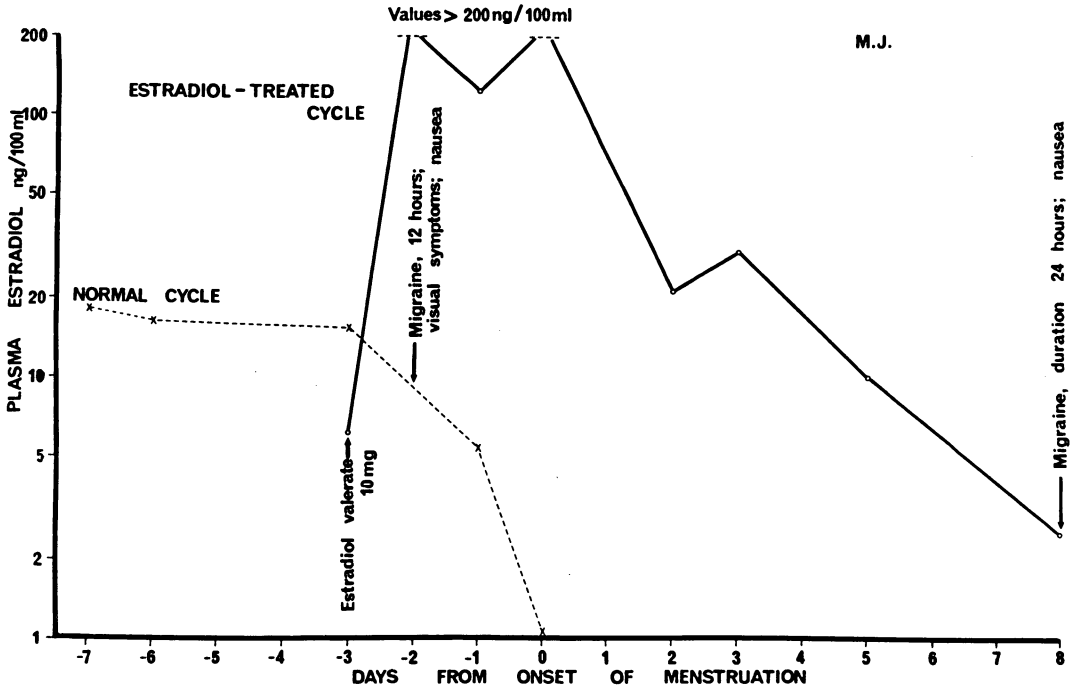

Values > 200 ng/100 ml
M.J.
ESTRADIOL - TREATED CYCLE
NORMAL CYCLE
PLASMA ESTRADIOL ng/100 ml
200
100
50
20
10
5
2
1
Migraine, 12 hours; visual symptoms; nausea
Estradiol valerate 10 mg
Migraine, duration 24 hours; nausea
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
DAYS FROM ONSET OF MENSTRUATION

Figure 8.6

FIGURES 8.7 TO 8.11

Plasma concentrations of progesterone and oestradiol during the oestradiol-treated cycles of the 6 migrainous women. It can be seen that, despite the maintenance of high levels of oestradiol, progesterone declined in the usual premenstrual pattern, and that this withdrawal of progesterone was not immediately followed by migraine.

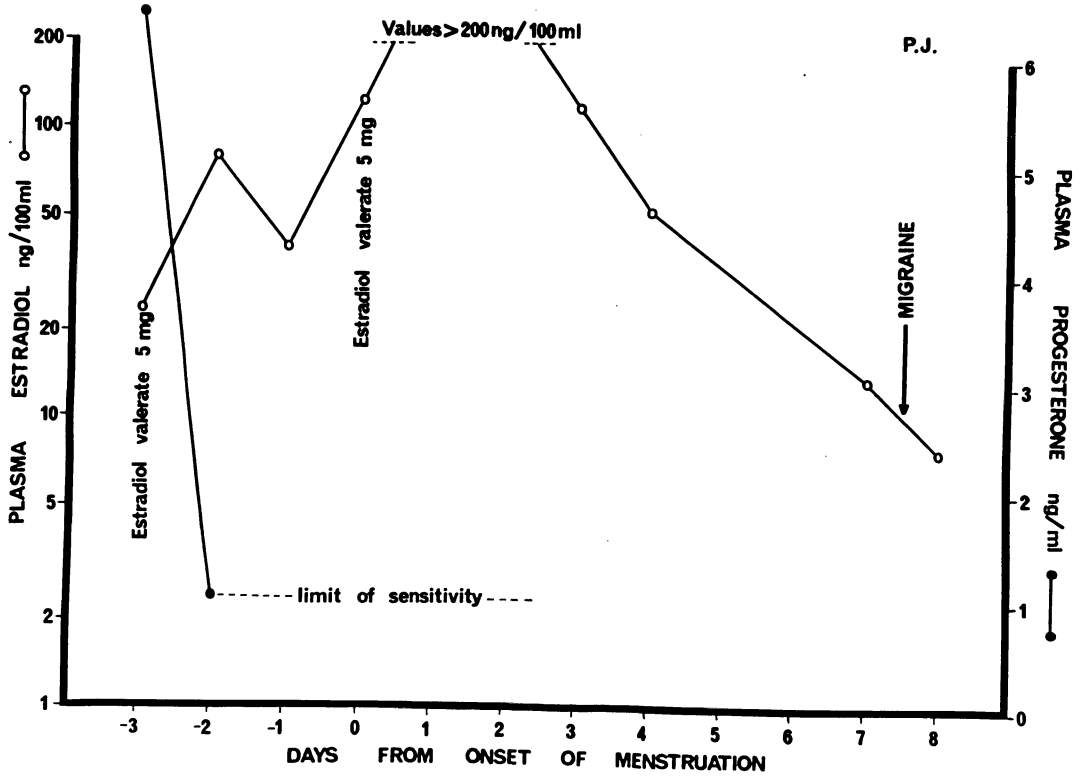

Values>200ng/100ml
P.J.
Estradiol valerate 5 mg
Estradiol valerate 5 mg
MIGRAINE
limit of sensitivity
PLASMA ESTRADIOL ng/100ml
PLASMA PROGESTERONE ng/ml
200
100
50
20
10
5
2
1
6
5
4
3
2
1
0
-3
-2
-1
0
1
2
3
4
5
6
7
8
DAYS FROM ONSET OF MENSTRUATION

Figure 8.7

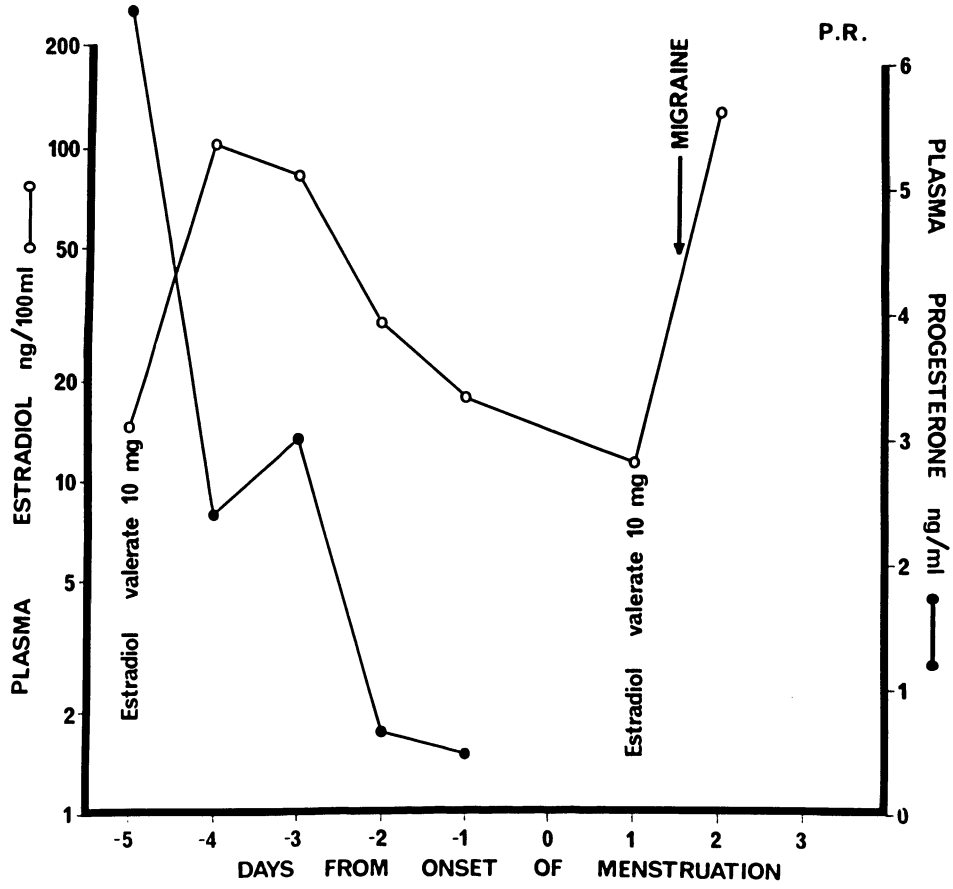

P.R.
PLASMA ESTRADIOL ng/100ml
PLASMA PROGESTERONE ng/ml
MIGRAINE
Estradiol valerate 10 mg
Estradiol valerate 10 mg
200
100
50
20
10
5
2
1
6
5
4
3
2
1
0
-5
-4
-3
-2
-1
0
1
2
3
DAYS FROM ONSET OF MENSTRUATION

Figure 8.8

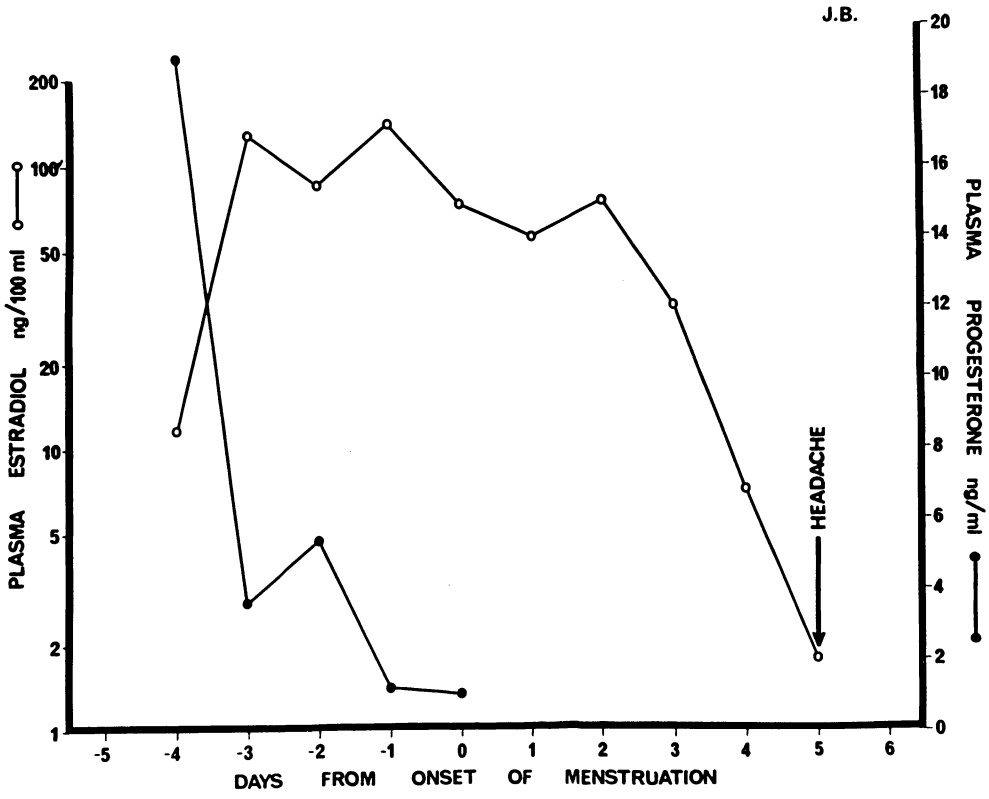

J.B.
PLASMA ESTRADIOL ng/100 ml
200
100
50
20
10
5
2
1
PLASMA PROGESTERONE ng/ml
20
18
16
14
12
10
8
6
4
2
0
HEADACHE
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
DAYS FROM ONSET OF MENSTRUATION

Figure 8.9

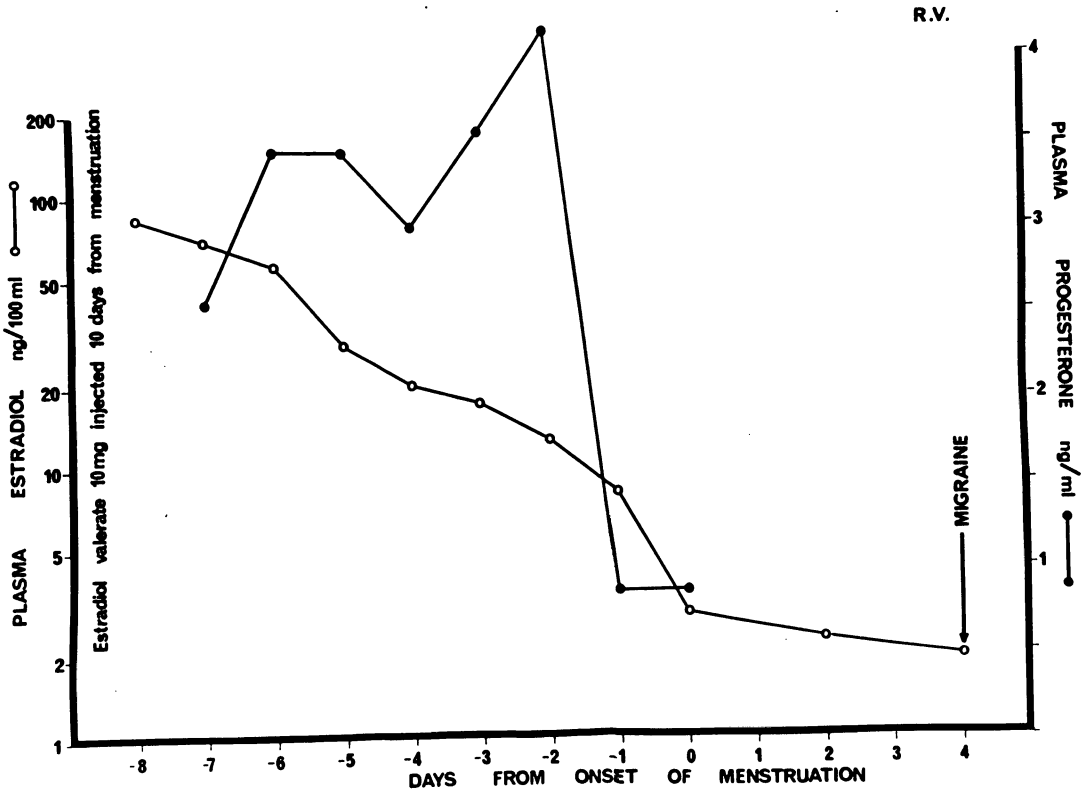

R.V.
PLASMA ESTRADIOL ng/100 ml
Estradiol valerate 10mg injected 10 days from menstruation
PLASMA PROGESTERONE ng/ml
MIGRAINE
200
100
50
20
10
5
2
1
4
3
2
1
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
DAYS FROM ONSET OF MENSTRUATION

Figure 8.10

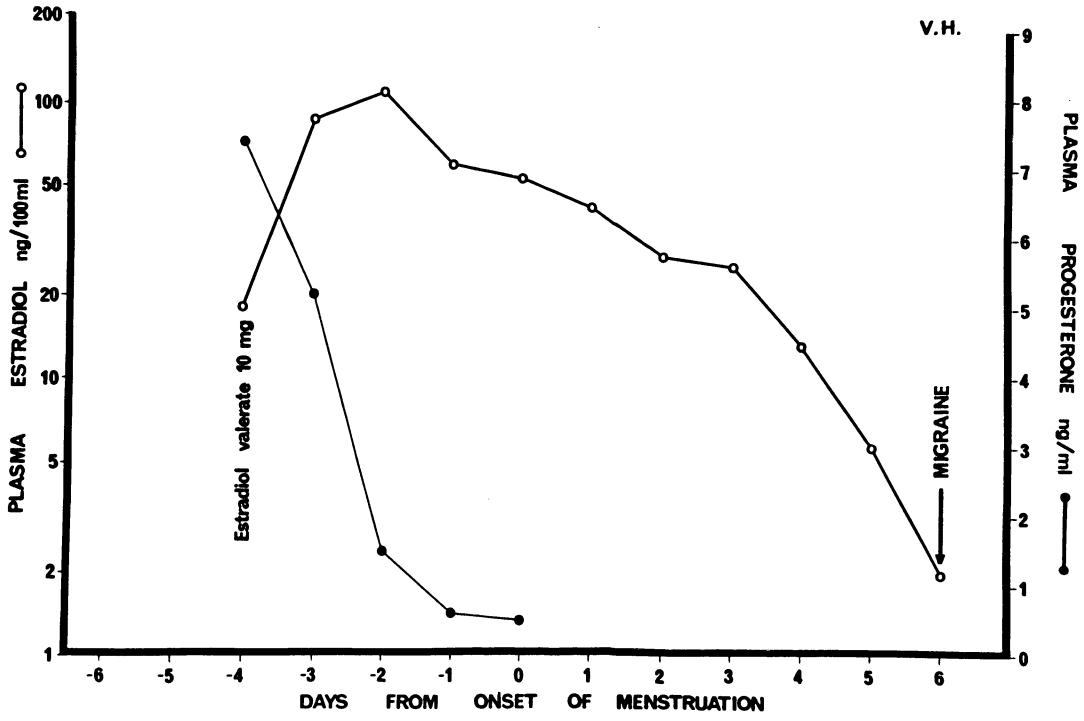
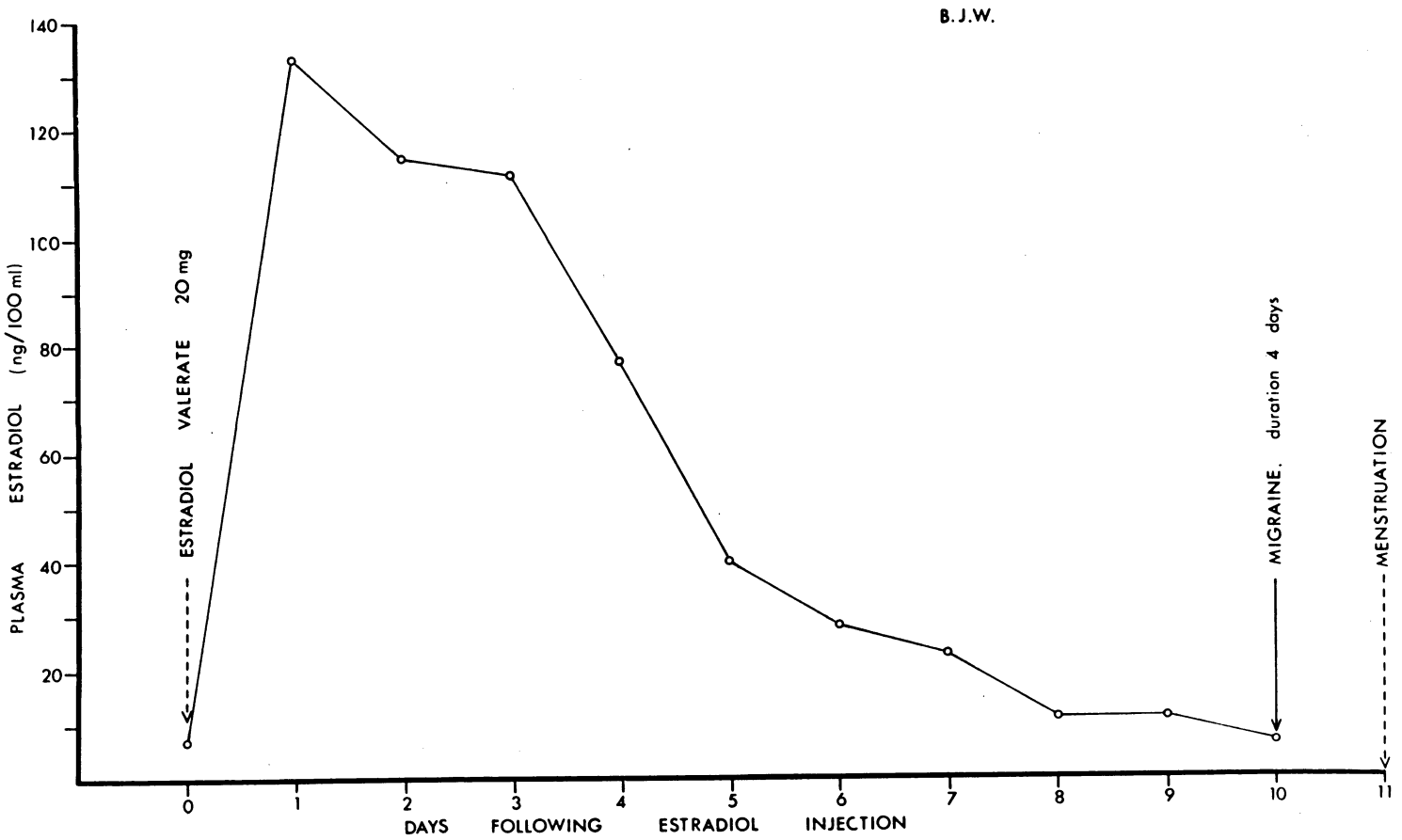

V.H.
PLASMA ESTRADIOL ng/100ml
PLASMA PROGESTERONE ng/ml
Estradiol valerate 10 mg
MIGRAINE
DAYS FROM ONSET OF MENSTRUATION
200
100
50
20
10
5
2
1
9
8
7
6
5
4
3
2
1
0
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6

Figure 8.11

FIGURES 8.12 AND 8.13

Plasma levels of oestradiol in women previously subject to regular menstrual migraine, whose menstrual cycles had ceased. Oestradiol levels immediately before, and after a single dose of oestradiol valerate are shown. Plasma progesterone levels did not rise above 2 ng/ml, and have not been shown. In both individuals, a typical migraine attack was provoked during the terminal phase of oestradiol withdrawal.

Figure 8.12


B.J.W.
PLASMA ESTRADIOL (ng/100 ml)
140
120
100
80
60
40
20
ESTRADIOL VALERATE 20 mg
MIGRAINE. duration 4 days
MENSTRUATION
0
1
2
3
4
5
6
7
8
9
10
11
DAYS FOLLOWING ESTRADIOL INJECTION

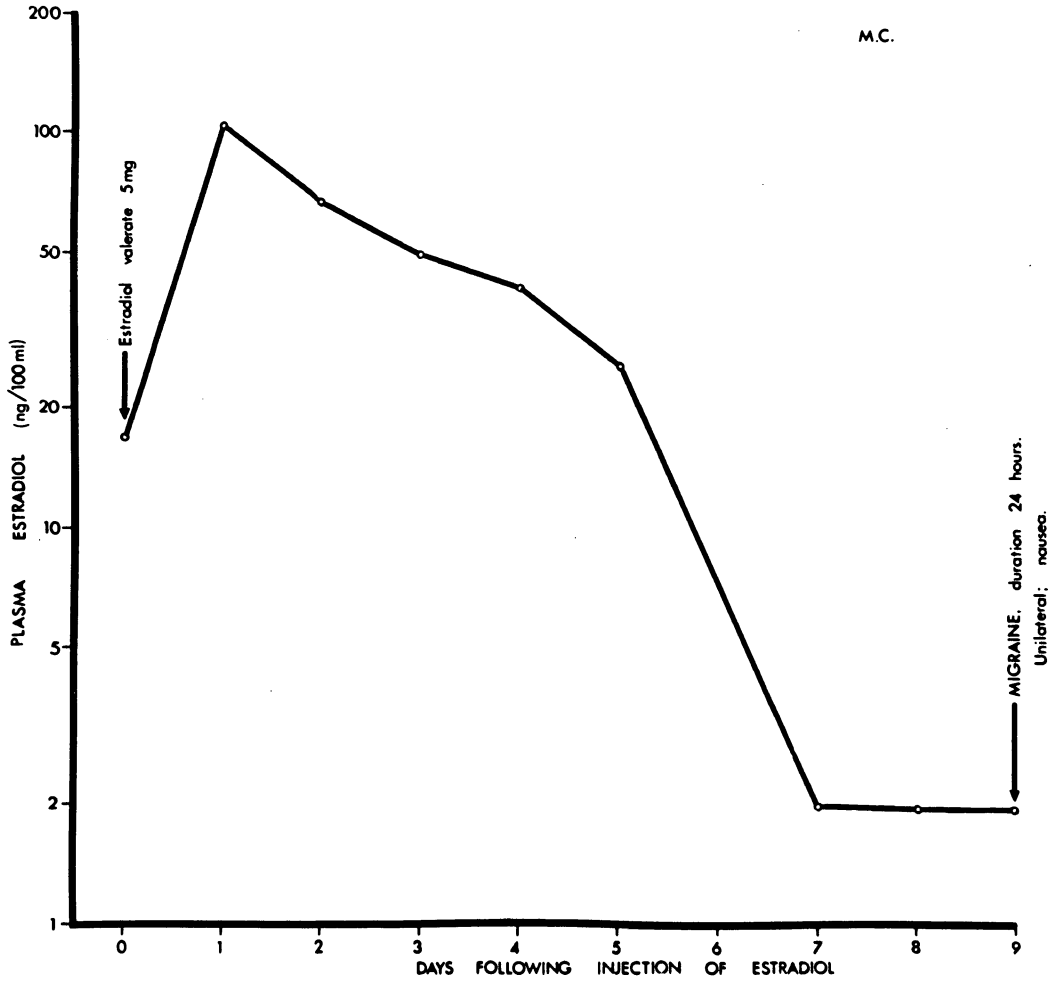

M.C.
200
100
50
20
10
5
2
1
PLASMA ESTRADIOL (ng/100 ml)
Estradiol valerate 5 mg
MIGRAINE, duration 24 hours.
Unilateral; nausea.
0
1
2
3
4
5
6
7
8
9
DAYS FOLLOWING INJECTION OF ESTRADIOL

Figure 8.13

Table 8.3

Plasma Oestradiol Concentrations in Two Women with Amenorrhoea, following the Administration of Oestradiol

Days following injection of oestradiol	Plasma oestradiol (ng/100ml) MRS. B.J.W.	MRS. M.C.
0	7.5	17.4
1	135.5	106.5
2	114.5	69.0
3	112.0	48.5
4	77.6	41.0
5	41.7	25.6
6	33.0	-
7	24.1	18.5
8	12.5	18.5
9	12.8	18.2
10	8.0	

Plasma progesterone was measured in each patient. In neither did the progesterone rise above 2 ng/ml. For this reason, progesterone values have not been included here.

Discussion

Five of the 6 regularly-menstruating women showed a similar response to the injection of oestradiol. While plasma oestradiol levels remained high (above 20 ng/100 ml) migraine was postponed. However, after oestradiol levels had fallen to lower values over the course of several days, each woman developed a migraine attack.

In the case of the sixth woman (Mrs. P.R., fig. 8.2), a second injection of oestradiol was administered on the sixth day, when the plasma oestradiol level had fallen to 12.1 ng/100 ml. This second injection did not prevent migraine, but the headache had obviously been delayed by the first injection. It would appear that, in this woman, the withdrawal of oestradiol had set in motion a series of changes which no longer responded to the later increase in plasma oestradiol caused by the second injection. This suggests that menstrual migraine may be caused by the withdrawal of a metabolite of oestradiol, such as oestrone; until further data concerning oestrogen levels in menstrual migraine become available, this question must remain speculative.

If any placebo effect were present, it would be expected to have caused relief, rather than postponement, of the migraine attack. Five of these 6 women had experienced migraine despite treatment with progesterone during previous cycles. This consistent pattern of delayed migraine observed in the oestradiol-treated cycles strongly suggests that the withdrawal of oestradiol plays a key role in the precipitation of menstrual migraine.

It can be seen from Tables 8.1 and 8.2 that during the oestradiol-treated cycle, progesterone withdrawal continued unaffected in each individual. This decline in progesterone levels was not accompanied by, nor immediately followed by migraine, and illustrates further that progesterone withdrawal per se does not appear to be capable of causing migraine.

In 3 of these subjects, the migraine attack was so delayed that it began after menstruation had ceased. This observation adds weight to the author's earlier suggestion that menstrual migraine does not depend upon uterine bleeding.

The possibility of a "threshold" plasma oestradiol concentration below which these women develop migraine, deserves brief consideration. When the onset of migraine

during normal and oestradiol-treated cycles is related to the plasma oestradiol concentration, there does not appear to be any clearly defined "threshold" value, either collectively, or in individual women. For example, Mrs. P.J. developed migraine during her control cycle when the plasma oestradiol was 1.8 ng/100 ml, while during the oestradiol-treated cycle, migraine began when the plasma oestradiol was as high as 9.5 ng/100 ml. (fig. 8.1).

In contrast, Mrs. R.V. experienced migraine during the control cycle at a plasma oestradiol level of 3.5 ng/100 ml, while during the oestradiol-treated cycle, migraine began at the lower level of 2.0 ng/100 ml (fig. 8.4). The absence of any clear "threshold" value of plasma oestradiol would appear to support the view that the withdrawal of oestradiol sets in motion a series of events which lead to the migraine attack. Thus, the absolute blood level of oestradiol appears to be of secondary importance compared to the process of withdrawal of the hormone from the circulation.

The two additional women who were not menstruating both developed their typical migraine attacks in response to oestradiol withdrawal. In one woman, oestradiol withdrawal did not cause uterine bleeding (Mrs. M.C., fig. 8.13). These women are of particular interest, in that they had

no significant fluctuations in the plasma progesterone level, which remained at the baseline. In their case, it appears difficult to attribute the headaches to any cause other than the withdrawal of oestrogen.

The mechanisms whereby the withdrawal of oestradiol might precipitate migraine will be discussed in Chapter 12.

SUMMARY

Oestradiol valerate was administered to 6 regularly menstruating women, who were subject to menstrual migraine, in such a way as to prevent the usual decline in plasma levels of the hormone premenstrually. Although menstruation was not delayed, the migraine attack was postponed in all women, by 3 to 9 days.

Similar treatment administered to 2 amenorrhoeic, migrainous women resulted in precipitation of migraine at the termination of the fall in plasma oestradiol.

It is concluded that the withdrawal of oestradiol sets in motion a series of changes which culminate in the development of migraine.

CHAPTER 9

VARIATION IN PLASMA SEROTONIN LEVELS MEASURED DAILY DURING THE PREMENSTRUAL AND MENSTRUAL PHASES, IN NORMAL AND MIGRAINOUS WOMEN

Introduction

Following the demonstration that plasma 5-hydroxytryptamine ("serotonin") falls sharply during the migraine attack (Curran, Hinterberger and Lance, 1965), and considering the known tendency for migraine to recur during or just before menstruation, it was decided to investigate plasma serotonin levels during these phases of the cycle. The level of blood serotonin was estimated daily in normal, non-migrainous women, and in women subject to regular menstrual migraine, to determine whether the clustering of migraine around this time of the cycle could be explained on the basis of altered blood serotonin levels.

Although wide day-to-day and diurnal fluctuations in the plasma level of serotonin are known to occur in the same individual (Curran et al., 1965), and, therefore, more frequent blood sampling would have been desirable, for technical reasons it was possible only to obtain single samples on successive days for 10-11 days.

Subjects

(a) Normal women: Five regularly menstruating women who did not suffer from migraine, were used as controls

(b) Migrainous women: Five regularly menstruating women, each prone to one predictable attack during the

premenstrual or menstrual phases of each cycle, were studied. The criteria for "migraine" have been listed above. No woman of either group was taking any hormonal preparation.

Methods

Daily samples of 20 ml of blood were obtained by venepuncture at approximately 9 a.m. Blood collections were commenced 5 days before the expected date of menstruation, and continued until 3 days after the onset of bleeding. Ten ml of each sample were used for the serotonin estimation, and the remaining 10 ml were used for hormone assays. Plasma serotonin was measured by the spectrofluorimetric method of Crawford and Rudd (1962).

Results

The daily plasma serotonin concentrations found during the premenstrual and menstrual phases in the 5 normal, non-migrainous women are shown in Table 9.1, and the values obtained in the 5 migrainous women are shown in Table 9.2. Statistical analysis of these figures shows that there was no significant variation in mean levels of serotonin during menstruation compared to the premenstrual period, although, as expected, highly significant variations in mean serotonin concentrations between individuals were observed.

Table 9.1 - Plasma Serotonin Levels in Normal, Non-Migrainous Women before and during Menstruation

Days from onset of menses	Plasma serotonin ($g/10^9$ platelets)				
	A.B.	S.B.	M.J.	M.S.	B.W.
-5	0.84	0.68	1.01	0.73	0.41
-4	0.89	0.91	0.84	0.65	0.66
-3	0.72	0.58	0.85	0.86	0.62
-2	1.04	0.66	0.89	0.83	0.64
-1	0.80	0.73	0.81	0.87	0.70
0	0.66	0.57	0.79	0.78	0.69
1	0.73	0.53	0.79	0.84	0.78
2	0.91	-	0.91	0.89	-
3	1.17	0.76	1.26	0.80	0.72

Table 9.2 - Plasma Serotonin Levels in women subject to Regular, Recurrent Menstrual Migraine

Days from onset of menses	Plasma serotonin (g/10^9 platelets)				
	M.J.	M.G.	E.K.	M.K.	J.C.
-5	0.96	0.64	0.67	1.52	0.48
-4	0.62	0.76	0.86	0.65	0.28
-3	0.59	0.54	0.83	0.99	0.35
-2	0.71*	0.53	0.57*	0.87*	-
-1	0.53	-	0.56	-	0.49*
0	1.10	0.47	0.68	0.91	0.21
1	0.72	0.46*	0.84	1.10	0.19
2	0.77	0.61	0.68	1.07	0.12
3	0.74	0.50	0.66	0.75	-

*Indicates the onset of migraine on that day

Discussion

There does not appear to be any consistent variation in plasma levels of serotonin in the days preceding or during menstruation in either migrainous or non-migrainous groups of women. The only similar relevant investigations in the literature appear to be those of Fuchs and Fuchs (1962), and Fuchs (1964), who measured the excretion of the main breakdown product of serotonin, 5-hydroxyindoleacetic acid (5-HIAA) in the urine throughout a number of normal menstrual cycles. The former authors found an ovulatory peak in the excretion of 5-HIAA, but could find no significant change in the rate of excretion of 5-HIAA before or during menstruation.

It should be pointed out that more frequent (e.g. 4-hourly) blood sampling, such as that employed by Curran et al. (1965) would be required to demonstrate the fall in serotonin during the migraine attack. In this study, serotonin levels in the migrainous subjects did not show any significant fall on the day of the attack.

Thus, the regular recurrence of migraine just before or during menstruation cannot be ascribed to any significant variation in plasma levels of serotonin, as measured by once-daily blood sampling.

CHAPTER 10

THE USE OF A CONTINUOUS PROGESTOGEN IN THE ATTEMPTED PROPHYLAXIS OF MIGRAINE

Introduction

Whitty et al. (1966) studied a number of patients who experienced migraine while taking oestrogen-progestogen combinations for suppression of ovulation. They observed that many of their patients suffered migraine regularly just before or during menstruation, presumably when plasma levels of the synthetic hormones were low or falling. They suggested that the trigger for the migraine attack could be withdrawal of the progestogen component, and observed that substitution of a lower dose of progestogen relieved migraine in some of their patients. They reported that 2 women, who had experienced menstrual migraine while taking oestrogen-progestogen combinations in cycles, were completely relieved of migraine when given these hormones continuously for several months.

Early in the course of the present work, it was considered that migraine attacks might therefore be abolished by suppressing the normal fluctuations in levels of female sex hormones. This may be achieved by the constant administration of a synthetic progestogenic hormone in a dose sufficient to suppress the hypothalamo-hypophyseal system. Previous reports of success in the prevention of migraine by the constant administration of

a synthetic progestogen, flumedroxone (6α-trifluoromethyl-17α-acetoxyprogesterone, "Demigran") from Lundberg (1969) and Bradley, Hudgson, Foster and Newell (1968) prompted the author to undertake a small preliminary trial using a constant contraceptive dose of the new synthetic progestogen, 3-acetoxychlormadinone ("AY 11440", Ayerst Laboratories, Montreal).

Methods

Twenty four patients attending the Outpatients' Department at the Royal Hospital for Women, Paddington, Sydney, took part in this preliminary trial. Both the investigator and the patients were aware that an active hormone preparation was being administered. Because of ethical considerations, and the desire of the patients for effective contraceptive protection, it was not possible to carry out a "double blind" type of investigation. Thus, it was not possible to eliminate either placebo effect or observer bias.

The definition of migraine, as stated earlier, was used in selecting patients for the trial. Patients were accepted only if they experienced at least one migraine per month over the past 6 months, whether or not this had been related to menstruation. The subjects were asked to keep a special

menstrual calendar, recording the basal body temperature, dates of bleeding, when a migraine attack occurred, and its severity and duration. In assessing the response to treatment, only the frequency of attacks was taken into consideration, as it was considered that the severity and duration were too subjective to be quantitated.

Each patient was instructed to take one tablet (0.8 mg) of AY 11440 at the same time each day. Patients were exam-ined subsequently at monthly intervals. The duration of follow up ranged from 3 to 7 months. The response was classified according to the frequency of attacks in the 6 months before the trial, compared with the frequency of attacks while taking the progestogen continuously. These 3 categories were made:

(a) No change: Frequency of migraine more than 50% of previous (control) frequency.

(b) 50%-improved: Frequency 50% or less than control.

(c) Headache free

To ensure that ovulation was being effectively suppressed, the plasma progesterone concentration was determined at some time during the 2 weeks before menstruation in each

cycle, using the competitive protein-binding system described in Chapter 4. In all cases, the plasma progesteron level was found to be less than 2 ng/ml. This low level was interpreted as confirming the absence of corpus luteum activity; i.e., ovulation had been effectively suppressed.

Results

The responses of the patients to the medication are shown in Table 10.1 The overall percentage of patients whose condition was improved to 50% or better was 41%.

Two patients were lost to follow up. One did not return after the initial interview, while in the case of the other, treatment had to be discontinued because of excessive irregular uterine bleeding.

Of the side effects produced, polymenorrhoea (decrease in the intermenstrual interval) was the commonest and most troublesome, and occurred in 19 out of the 22 women (86%). Breakthrough bleeding, ranging from slight intermenstrual spotting to prolonged bleeding, occurred in 14 women (64%). Complete amenorrhoea developed in 3 women. No pregnancies occurred. The incidence and nature of side effects are shown in Table 10.2

Table 10.1- Clinical Response of 22 women treated with a continuous daily dose of AY 11440, 0.8 mg

	Number of cases		
	No Change	50% Improved	Headache free
Migraine existing before taking oral contraceptives			
(a) Menstrual	6	3	1
(b) Non-menstrual	4	2	0
Migraine commencing with oral contracep--tive therapy			
(a) Menstrual	2	1	0
(b) Non-menstrual	1	1	1

Table 10.2- Incidence of Side Effects in 22 Women

Polymenorrhoea	19	Abdominal swelling	1
Intermenstrual bleeding	14	Nausea	2
Depression	4	Acne	1
Amenorrhoea	3		
Mastalgia	3		

Discussion

The results of this preliminary trial were disappointing. The overall rate of improvement was 41%. Previous double-blind trials of drugs in the prevention of migraine carried out at Prince Henry Hospital have shown a placebo effect ranging from 20% to 36% (Anthony and Lance, 1968; Lance and Anthony, 1968). Although a direct comparison of the present series with these other studies cannot be made, it would appear than any prophylactic effect of the medication in this trial must have been minimal. The setting up of a double-blind controlled trial was not considered to be justified on the basis of this study.

Continuous progestogen contraception was introduced by Rudel (1965). The original aim was not to interfere with gonadotrophin secretion, but to produce local changes in the female genital tract (especially alteration of the cervical mucus) which would provide a reliable contraceptive effect. Widespread experience during the last 5 years has shown that the rate of failure for this form of contraception is sub-stantially higher than that of the standard oestrogen-progestogen combinations (Annotation, Lancet, 1969). The reliability of the method can be increased by raising the dose of progestogen to the level at which the hypothalamo-hypophyseal system is suppressed. At these higher dose levels (such as those of the present study), troublesome

side effects, especially amenorrhoea and menstrual irregularities, usually appear.

Although there did not seem to be any distinct benefit from continuously administered progestogen in this trial, it was observed that, in those women who experienced intermenstrual bleeding, a high proportion (9 out of 14) suffered migraine during such bleeding, even though in many cases, the amount of bleeding was very slight. In this context, it is noteworthy that the 2 women who became completely free of headaches while on the treatment also became amenorrhoeic.

By measuring the level of endogenous progesterone, it was established that ovulation had been suppressed in all patients. The migraine associated with intermenstrual bleeding could hardly have been caused by the withdrawal of progestogen, since these women were receiving a constant daily dose, and were producing only baseline levels of natural progesterone. A more likely explanation for the simultaneous occurrence of breakthrough bleeding and migraine appears to be fluctuation in oestrogen levels, which have been shown to occur even when ovulation is suppressed (Shearman, 1964).

Attempts at preventing migraine by continuous administration of progestogenic hormones were first stimulated by reports of success in using natural, injected progesterone. Lundberg (1962) initially used methyl nor-testosterone and allyloestrenol in treating 76 women and 8 men suffering from migraine, and reported striking success (freedom from headache in 49 women and 6 men). The same author reported a similar rate of success in a number of trials (one conducted as a double-blind cross-over trial), using a newer synthetic progestogen, flumedroxone (6α-trifluoromethyl-17α-acetoxyprogesterone). This steroid is claimed to have fewer side effects owing to its weaker systemic progestational effects.

Other workers have failed to achieve comparable results with flumedroxone. Thus, in a double-blind cross-over trial in which flumedroxone was compared with methysergide, the latter drug emerged as clearly superior (Hudgson et al., 1967). The same group subjected a micronised preparation of flumedroxone to a further double-blind trial, and found it to be significantly better than placebo in the treatment of women suffering from menstrual migraine, although the order of improvement was not impressive. Flumedroxone was not significantly better than placebo in the treatment of either women suffering from non-menstrual migraine, or males. A larger double-blind trial conducted

in Denmark failed to show any advantage of the drug when compared to placebo (Thygesen, Klee and Fog, 1969).

A universal finding among workers using continuous progestogens has been a high incidence of menstrual irregularities. Although it is generally assumed that the beneficial effect of synthetic progestogens in migraine is due to a direct effect upon the cranial vessels, it would appear more likely, in the light of the present study, that the prophylactic effect of these hormones, such as it is, is due to a suppression of the normal fluctuations in plasma levels of oestrogens, secondary to inhibition of pituitary gonadotrophin secretion caused by the synthetic hormone.

Headache as a side effect of oestrogen-progestogen combinations was first reported by Mears and Grant (1962). Subsequently, several workers have provided convincing evidence of a causal relationship between the taking of these hormone combinations and migraine (e.g. Shafey and Scheinberg, 1966; Whitty et al., 1966; and Phillips, 1968). Although the usual effect has been an exacerbation of pre-existing migraine, or the appearance of migraine de novo, the opposite effect of improvement of migraine in a minority of cases has been reported (Gispert-Cruz and Girons, 1967; Larsson-Cohn and Lundberg, 1970). Indeed, in a large "open" trial conducted by the latter workers,

in which the frequency of migraine was studied in women on combined or sequential oestrogen-progestogen mixtures with a low dose of progestogen, it was found that migraine was significantly improved in women who had suffered from migraine before beginning the treatment.

The progestogenic component of the earlier oral contraceptives was of the C-19 nor configuration, and these compounds are known to be converted metabolically into oestrogens to a significant extent. For this reason, the current trial employed a 17-acetoxyprogesterone, since steroids of this configuration are not metabolised to oestrogen. It is possible that the effect of withdrawal of progestogens of the C-19 nor variety would indeed precipitate migraine, through the withdrawal of oestrogenic metabolites formed from these progestogens.

SUMMARY

A small pilot trial of the use of the continuous progestogen contraceptive, 3-acetoxychlormadinone (AY 11440) in dosage of 0.8 mg daily, in the attempted prevention of migraine is described. Twenty two patients were followed for from 3 to 7 months. The condition of 9 patients showed an improvement to 50% or better. The incidence of

side effects, particularly polymenorrhoea and breakthrough bleeding, was high. It was concluded that the benefit of such prophylactic treatment, if any, must be small, and is probably outweighed by the high incidence of side effects.

The close relationship between uterine bleeding and migraine in some patients was noted, and the mechanism of this was suggested to be fluctuation in plasma oestrogen levels. It was further suggested that any beneficial effect on migraine of continuous administration of synthetic progestogens may be due to suppression of the normal fluctuations in levels of female sex hormone secondary to inhibition of pituitary gonadotrophin secretion

CHAPTER II

A STUDY OF MIGRAINE DURING PREGNANCY

INTRODUCTION

During pregnancy, profound changes take place in the hormonal milieu. These changes are evident as early as 8-10 days after fertilisation, and continue well after delivery, until lactation ceases, and the normal menstrual pattern returns. The development of the placenta as a functioning endocrine organ, and the ability of the foetus to produce and metabolise steroid hormones are major factors in altering the pattern of oestradiol and progesterone in the maternal circulation.

Quantitatively, changes in plasma concentrations of oestradiol and progesterone are dramatic - during late pregnancy, the concentration of oestradiol is increased to the order of 100 times compared to non-pregnancy levels. It would be surprising, therefore, if pregnancy did not exert a modifying influence on migraine attacks. In fact, pregnancy does alter the pattern of migraine in most women.

It has been accepted generally that pregnancy is associated with either complete freedom from migraine, or a decrease in the frequency of attacks (Critchley and Ferguson, 1933; Friedman and Merritt, 1959). More recently, however, one clinical study has suggested the opposite view, i.e., that migraine is frequently exacerbated by

pregnancy, and that migraine often begins during pregnancy (Callaghan, 1968). In view of these apparently contra--dictory reports, the current study was undertaken in an attempt to resolve the question.

This study consists, therefore, of a clinical survey similar in design to Callaghan's study, in which 200 women attending the antenatal outpatients' clinic of a large general hospital in Dublin were questioned concerning headaches. In addition to interviewing a similar number of women in Sydney, the plasma concentration of progesterone was determined in groups of women to determine whether relief from migraine, or alternatively, failure to obtain relief from migraine during pregnancy could be correlated with differences in the plasma level of progesterone.

METHODS

Two hundred pregnant women attending the routine antenatal clinic at the Women's Hospital, Crown St., Sydney, were questioned on one occasion during the 4 weeks before the expected date of confinement. These women were mainly from the lower socio-economic groups of an urban environment. Women were excluded from the study if the duration of pregnancy were in doubt. Many women, mainly newly arrived immigrants from southern Europe, were excluded because of language difficulties.

It was decided to interview the women during the 36th to 40th weeks, since plasma progesterone levels do not show significant elevation during this period (Johansson, 1969).

Each woman was questioned concerning headaches. The minimal criteria for a diagnosis of migraine were: recurrent, paroxysmal headaches, not necessarily unilateral, but either associated with nausea or visual symptoms (blurred vision, photopsia, fortification spectra), or sensory or motor symptoms or dysphasia. The minimum duration accepted was 1 hour. Although some degree of photophobia was usually present, its absence did not preclude the diagnosis of migraine.

Details of migraine attacks preceding and during the current pregnancy were recorded, and included the frequency and duration of attacks, the site of the headaches, and the presence of any associated visual, sensory or motor symptoms.

A single 10 ml blood sample was obtained from women of the following categories:

(a) Group A: Non migrainous women (controls)

(b) Group B: Women suffering from migraine before pregnancy, in whom migraine attacks had improved during pregnancy.

(c) Group C: Women who had suffered migraine before pregnancy, who had failed to experience relief.

All blood samples were taken during the 4 weeks before the expected date of confinement. Progesterone was assayed as described above, using duplicate samples of 0.05 ml plasma The thin-layer chromatographic step was employed with all pregnancy plasmas, and the recovery of ^{3}H-progesterone was used to provide an appropriate correction for procedural losses in each sample.

Classification of patients: Patients were classified as "migrainous" or otherwise according to whether or not they had been subject to migraine attacks in the 12 months before becoming pregnant. Women who gave a more distant history of migraine, often in childhood or their teens, but who had not suffered from migraine in the 12 months before conception were classified with the "non-migrainous" group (A). Women who had been subject to migraine attacks within 12 months of becoming pregnant were further divided into groups according to whether the migraine frequency had improved (Group B), or had remained unchanged or had worsened during pregnancy (Group C). "Improvement" was decided solely on the basis of frequency of attacks, since it was felt that severity was too subjective a symptom to quantitate accurately. Patients were classified as "improved" if the frequency of attacks were reduced to 50% or less than that prevailing before pregnancy.

RESULTS

A. Clinical Study

Of the 200 women questioned, 38 gave a recent history of migraine. Of these, 31 had been subject to migraine before becoming pregnant, and 7 had developed migraine for the first time during the current pregnancy. In this latter group, migraine began in the first trimester in 5 women, in the second trimester in one, and in the third trimester in the remaining woman. In the case of the 31 women who had been migraine sufferers at the time of falling pregnant, 24 showed improvement (including 7 who were completely free of headaches throughout the pregnancy), while the remaining 7 women either failed to improve, or deteriorated.

Table 11.1 summarises these results, and compares them to the figures obtained by Callaghan (1968).

B. Plasma Progesterone Determinations

Table 11.2 shows plasma progesterone concentrations (ng/ml) in women during the 4 weeks before the expected date of delivery. These values were obtained by performing assays on a single plasma sample taken during this period. They have been tabulated in Groups A, B, and C as defined above. Statistical analysis reveals that there is no significant difference in mean values between these groups (see statistical appendix).

Table 11.1- Comparison of Results in two series of 200 Pregnant Women questioned concerning Migraine

	Somerville, 1970 (current study)	Callaghan, 1968*
Women experiencing attacks during current pregnancy	38	41 (25 + 16)
Attacks before becoming pregnant		
(a) Total	31	8 (5 + 3)
(b) "Improved"	24	4 (3 + 1)
(c) Unchanged or worse	7	3 (2 + 1)
Attacks beginning during this pregnancy	7	33 (20 + 13)

*Callaghan's figures refer to the total numbers of patients suffering from "true migraine" and "probable migraine" combined. The numbers in each respective category are shown in parentheses following each total.

Table 11.2 - Plasma Progesterone Levels (ng/ml) in Pregnancy

GROUP A (controls)	GROUP B (relieved)	GROUP C (unrelieved)
72.4	75.5	175.0
78.0	85.5	67.5
160.5	101.0	102.5
102.0	170.0	78.0
129.0	124.0	88.9
106.0	97.5	95.5
195.0	80.0	
98.5	71.1	
71.0	104.5	
167.5	84.5	
95.0	88.9	
80.0		
143.0		
99.0		
79.0		
88.0		
132.0		
167.0		
160.0		
163.0		
Mean (S.E.) 119.38 (8.67)	98.41 (8.47)	101.23 (15.6)

DISCUSSION

A. Clinical Study

The clinical part of this study was designed along lines similar to those of Callaghan (1968), so that some broad comparison of results might be possible. Several factors preclude a direct comparison of results between the two series, however; the most important of these is probably the lack of definition of what constituted "improvement" in the Dublin series. Callaghan divided his migrainous patients into two groups. Those in whom unilateral headaches were associated with any two of the following symptoms: nausea, vomiting, blurred vision, fortification spectra, diplopia, transient hemiparesis or hemianaesthesia, or speech disturbance, he classified as suffering from "true migraine"; while those suffering from bitemporal headaches, or unilateral frontal headaches were classified as "probable migraine". Comparing the criteria for migraine used in the present study, it seems likely that several of the patients classified by Callaghan as suffering from "probable migraine" would not have been classified as migrainous in the current series.

Although both studies concerned women of the lower socio-economic groups, there obviously exist large differences in ethnic background, climate, dietary habits, and many other factors between the two populations of women sampled.

Within these limitations, it is still of interest to compare the figures obtained. The principal differences between the series appear to be:

(a) Prevalence of migraine: Callaghan found that only 4% of the women he studied had suffered migraine before conception, while in the present series, the prevalence was 16%. This difference becomes even more pronounced when it is remembered that the criteria for a diagnosis of migraine appear to be stricter in the latter study.

Estimates of the prevalence of migraine vary widely. In a recent survey of the prevalence of migraine in the general population, Waters and O'Connor (1969) found that the figure for women aged 20 to 64 was 19%. In another recent epidemiological survey conducted in Denmark, it was found that the prevalence of migraine in women aged 40 was 19% also (Dalsgaard-Nielsen, 1969).

(b) Clinical Course of Migraine during Pregnancy: A higher proportion of migrainous Australian women (77%, 24 out of 31) obtained improvement compared to their Irish counterparts (50%, 4 out of 8), although the absolute numbers are probably too small to make comparison meaningful

(c) Appearance of migraine de novo during pregnancy

The difference between the series concerning this point is marked. In Callaghan's series, as many as 33 women experienced migraine for the first time during pregnancy, whereas the number was only 7 in the current study. This discrepancy may be explained in part by the more liberal definition of "migraine" in the former series.

There is some agreement evident, however, in that when migraine does develop for the first time during pregnancy, it most often appears in the first trimester (16 out of 33 in the Dublin series, 5 out of 7 in the Australian series).

The current study appears to confirm the traditional view that migraine generally improves during pregnancy. Friedman and Merritt (1959) state that 80% of migrainous women experience complete or partial relief from migraine during pregnancy. Lundberg (1962) found that 35 out of 40 women experienced improvement or freedom from migraine during pregnancy. The order of improvement found in the current study (77%) is comparable to these figures, and emphasises the improvement in migraine which usually occurs in pregnancy.

This work has, however, lent some support to Callaghan's claim that migraine may be aggravated by pregnancy, or may appear de novo during this time, although there is a wide

variation in the frequency with which this was observed in the two series.

One noteworthy finding during the current study was a high incidence of non-migrainous, vascular-type headaches, largely confined to the first trimester. These headaches were usually bifrontal and throbbing in character, and often recurred daily for several days to weeks. Because of the lack of any associated migrainous symptoms, and the frequently mild nature of these headaches, no clear cut distinction between these and tension headaches could always be drawn. Accordingly, it was not possible to collect data concerning the incidence of these headaches.

B. Progesterone Estimations

The progesterone values obtained here agree well with those reported by other workers (e.g., Johansson, 1969). The mean plasma progesterone concentration in those women who obtained relief did not differ significantly from the mean value of the women who did not obtain relief, nor from the mean value of normal, non-migrainous pregnant women. Therefore, it would appear that relief from migraine during pregnancy does not depend upon any absolute level of progesterone in the blood, so that the failure of some women to obtain relief during pregnancy cannot be attributed to a lower plasma level of progesterone conferring an inadequate

"protective" effect against migraine.

It is clear from the results of the current study and of other workers that the clinical course of migraine is usually significantly altered by pregnancy. Some of the mechanisms whereby this might occur will be briefly considered. These include:

(a) Suppression of fluctuations in sex hormone levels.

(b) Inhibition of vasomotor responses of the cranial arteries by the raised levels of oestrogens, or of progesterone, or of both oestrogens and progesterone

(c) Alteration of the angiotensin-renin-aldosterone mechanism.

Suppression of fluctuations in sex hormone levels

The rise and fall of plasma levels of progesterone and 17α-hydroxyprogesterone during the waxing and waning of the corpus luteum during early pregnancy have been described previously. At the risk of making an oversimplif--ication, this early period of hormonal "turbulence" might be expected to aggravate migraine, through the effects of a rising, then falling pattern of progesterone and 17α-hydroxyprogesterone against a background of rapidly increasing plasma levels of the three classical oestrogens

affecting a susceptible cranial vasculature. It has been shown that in non-pregnant women subject to menstrual migraine, a withdrawal of oestradiol or its metabolites may precipitate migraine. It is possible that the falling levels of metabolites of progesterone during early pregnancy may aggravate migraine also, but in the absence of data concerning the vascular effects of these metabolites, this possibility must remain conjectural.

The observation that the majority of women experience relief from migraine during the second and third trimesters, when progesterone and oestrogen levels are steadily rising, suggests that migraine may be improved through the elimination of rapid rises or falls in hormone levels which occur during the normal menstrual cycle. Such a mechanism may also be suggested to explain the relief of migraine which usually follows the menopause, although in the latter condition, hormone levels remain low after ovarian function has completely ceased.

Inhibition of vasomotor responses of the cranial arteries by raised levels of oestradiol or progesterone

The classic studies of Wolff (1963) have shown that the migraine attack is characterised by a preliminary phase of intracerebral vasoconstriction, followed by a painful dilatation of the affected scalp arteries. It is possible

that the elevated hormone levels of pregnancy suppress these abnormal vascular changes, and thereby confer protection against the migraine attack.

Progesterone has been shown to have a hypotensive effect when administered to non-pregnant women in large doses (De Soldati and Suarez-Navas, 1962). McCausland, Holmes and Trotter (1963) have shown that venous distensibility measured in the digital vessels follows closely the progest--erone curve, and is greatest one week premenstrually. These observations appear to suggest that progesterone has an inhibitory effect upon vascular smooth muscle. Whether this effect is a direct one, or is mediated through neural mechanisms, is unknown.

If the greatly elevated level of progesterone in plasma during pregnancy were responsible for the clinical improve--ment usually observed in migraine, one might expect that the mean level of progesterone in migraine sufferers who fail to obtain relief in pregnancy would be significantly lower than the mean level in those migrainous women whose migraine showed improvement in pregnancy. Such a relationship was not demonstrable, however, in the current study.

Alteration of the angiotensin-renin-aldosterone mechanism

Pregnancy is associated with an increase in the secretion of aldosterone (Vande Wiele, Gurpide, Kelly, Laragh and Lieberman, 1960). This increase may be compensatory, for progesterone antagonises the effect of aldosterone on the renal tubules, and promotes the excretion of sodium (Landau and Lugibihl, 1961). The increased loss of sodium is probably responsible for the rise in plasma levels of renin which is observed during the progesterone-dominated phase of the menstrual cycle (Brown, Davies, Lever and Robertson, 1964), and during pregnancy (Genest, de Champlain, Veyrat, Boucher, Tremblay, Strong, Koiw, and Marc-Aurele, 1965). The level of renin activity in late pregnancy is of the order of 12 times greater than the non-pregnancy level, and is due to elevations both of renin and renin-substrate (Lumbers, 1970).

This greatly increased renin activity would be expected to cause a rise in plasma levels of angiotensin in pregnancy. The pressor effect of any such rise could be offset by two factors. The first is an increase in the level of angiotensin-inactivating enzymes, which occurs in pregnancy (Talledo, Rhodes and Livingstone, 1967). The second is a "blunting" of vascular responses to angiotensin, which has been demonstrated in pregnant women by measuring the change

in forearm skin blood flow in response to intravenous infusions of angiotensin. The reduction in response to angiotensin was not found to be accompanied by a similar reduction in response to nor-adrenaline (Lumbers, 1970), and does not appear to be caused by elevated plasma progesterone (Chesley and Tepper, 1967).

It has been suggested that raised plasma renin levels may be responsible for the increased incidence of migraine in women taking oestrogen-progestogen combinations (Greene and Stanford, 1969). Since pregnancy is associated with a greatly increased plasma level of renin (Genest et al., 1965) and an increased secretion of aldosterone, its beneficial effect on migraine cannot be explained on this basis. The possible role of aldosterone in menstrual migraine will be considered in Chapter 12.

The suggested relationship between migraine and pre-eclamptic toxaemia deserves mention. Clinical observations by Rotton, Sachtleben and Friedman (1959) led these authors to propose that migraine attacks occur more frequently in pregnant patients who develop pre-eclampsia than in normal pregnant women. From their retrospective clinical study, they suggested that failure to obtain relief from migraine during pregnancy implied an increased liability

to pre-eclampsia. The current study was not designed to confirm or refute their conclusions, but it is of interest that the 7 women whose migraine had deteriorated or failed to show improvement did not evidence any sign of pre-eclampsia when examined clinically in the four weeks before the expected date of confinement. It should be noted, however, that it is the practice at the Women's Hospital to admit patients with pre-eclampsia, so that the migrainous women who developed this condition would not have been available for interview in the routine antenatal clinic.

While the aetiology of pre-eclamptic toxaemia remains obscure, it would be fruitless to speculate on possible mechanisms whereby this condition might be related to migraine. When the incidence of both conditions are considered, it is clear that critical statistical studies will be required to establish whether any relation exists between the two disorders.

SUMMARY

A survey of migraine in pregnancy, involving the questioning of 200 pregnant women attending the antenatal clinic at the Women's Hospital, Crown St., Sydney, has been carried out. Women were interviewed during the four weeks before the expected date of confinement, and at this time, blood samples were obtained for plasma progesterone determin-ation.

Of the 200 women questioned, 38 gave a recent history of migraine. Of these, 31 had been subject to migraine before becoming pregnant, and 7 had developed migraine for the first time during the current pregnancy. In this latter group, migraine began in the first trimester in 5 women, in the second trimester in one, and in the third trimester in the remaining woman. In the case of the 31 women who had been migraine sufferers at the time of falling pregnant, 24 showed improvement (including 7 who were completely free of headaches throughout the pregnancy), while the remaining 7 women either failed to improve, or deteriorated.

No significant difference in mean plasma progesterone levels was observed between groups of normal, non-migrainous pregnant women, migraine sufferers whose migraine had improved during pregnancy, and migrainous women who had failed to improve during pregnancy.

CHAPTER 12

CORRELATION OF PREVIOUS KNOWLEDGE WITH FACTS ESTABLISHED BY THIS WORK, AND FORMULATION OF HYPOTHESIS

The underlying cause for the unique combination of vascular changes which comprise the migraine syndrome remains obscure. The clinical picture of a paroxysmal, usually unilateral headache, lasting for hours to days at a time, associated with nausea and photophobia, and preceded or accompanied by visual, sensory or motor symptoms or dysphasia, is a distinctive one. While the basic defect which renders the cranial vessels liable to undergo these changes is not understood, research in recent years has been directed toward the study of factors which may precipitate the migraine attack.

To date, the evidence linking migraine with changes in female sex hormones has been mainly circumstantial. It is clear that such hormonal changes cannot be the underlying cause of migraine, since one in four sufferers is a male. Nevertheless, the following facts clearly indicate that migraine may be influenced strongly by changes in female hormones:

1. Association of migraine with menstruation

The syndrome of premenstrual or menstrual migraine is well recognised. In this disorder, migraine recurs with striking regularity only during a specific phase of the menstrual cycle. According to de Wit (1950), such an association can be recognised in 60% of women suffering

from migraine. Lundberg (1962) has emphasised that migraine frequently begins at puberty, and usually remits after the menopause.

2. Modification of migraine by pregnancy

The usually beneficial effect of pregnancy upon migraine (Critchley and Ferguson, 1933) has been confirmed by the study described in Chapter 11, although the opposite effect of aggravation of migraine in some women has been described (Callaghan, 1968). Whether migraine shows the usual improvement or otherwise, there can be no doubt that pregnancy usually exerts an important influence on migraine.

3. Relation between migraine and oral contraceptives

Since headache was first reported as a side effect of oestrogen-progestogen combinations by Mears and Grant in 1962, many workers have found a clinical association between the taking of these drugs and the exacerbation of migraine. It is now generally accepted that there is a causal relationship between the taking of oestrogen-progestogen contraceptives and migraine.

4. Preponderance of migraine in women.

Although figures for the prevalence of migraine and the ratio of female to male sufferers vary widely, there is general agreement that women are more prone to

the disorder than men. In a recent study of the prevalence of migraine in Denmark, Dalsgaard-Nielsen, Engberg-Pedersen and Holm (1970) found a female: male ratio approaching 1:2 at age 40.

In addition to this evidence implicating female hormones in migraine, there is reason to believe that changes in the levels of other vasoactive substances are important in the aetiology of migraine. These will now be considered.

5-Hydroxytryptamine (5-HT, Serotonin) and Migraine

Sicuteri (1961) demonstrated that the migraine attack is accompanied by an increased urinary excretion of the major metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA). Subsequently, Curran, Hinterberger and Lance (1965) found that the plasma level of serotonin falls during migraine. It has been suggested that this sudden fall in plasma serotonin results in dilatation of the scalp arteries, and therefore initiates the migraine attack (Anthony, Hinterberger and Lance, 1969). The drop in plasma serotonin is due to the release of serotonin from platelets, which appears to be caused by a "serotonin-releasing factor" appearing at the time of the migraine attack (Anthony et al., 1969).

The clinical benefits derived from the use of drugs with serotonin-antagonising properties in the prevention of migraine emphasise the importance of serotonin metabolism in the migraine syndrome. These drugs include methysergide, cyproheptadine, and BC 105 (Lance, Anthony and Somerville, 1970).

Tyramine and "Dietary" Migraine

Further evidence emphasising the role of vasoactive amines in migraine has been provided by the studies of Hannington (1967, 1969) who showed that migraine may be precipitated in some patients suffering from "dietary" migraine (i.e., migraine regularly precipitated by some article of diet, such as chocolate or cheese) by the administration of tyramine. Smith, Kellow and Hannington (1970) studied the urinary excretion of tyramine in normal controls and in patients suffering from "dietary" migraine before and after an oral dose of tyramine, and found that the migrainous patients excreted significantly less free and conjugated tyramine both before and after oral loading with tyramine.

They suggested that there is a disorder of aromatic amino acid metabolism in patients suffering from "dietary" migraine, although they were unable to define the exact nature of the defect.

Fluid Retention, Aldosterone, and Migraine

The role of fluid retention in the pathogenesis of migraine has excited interest ever since Quincke in 1897 drew attention to the association between migraine and oedema. Fluid retention during the premenstrual phase is common both in migrainous and normal women, and may be due to the increased secretion of aldosterone which is observed during the second half of the cycle (Gray, Strausfeld, Watanabe, Sims and Solomon, 1968), or to the salt-retaining properties of oestradiol (Thorn and Engel, 1938).

Schottstaedt and Wolff (1953) and Wolff, Ostfeld, Reis and Goodell (1955) confirmed that considerable fluid retention precedes menstrual migraine in many women, but found that the prevention of fluid retention by restriction of fluid intake does not prevent the usual migraine attack. They could not precipitate migraine by treating migrainous subjects with fluid retaining hormones (including pitressin and deoxycorticosterone acetate). Their conclusion was that although salt and water retention frequently precede migraine, there is no evidence that this is the cause of migraine. Their conclusions have been supported by reports of the clinical ineffectiveness of diuretics in attempting to prevent menstrual migraine (Diddle, Gardner and Williamson, 1969).

The case of a patient with long standing migraine and Conn's syndrome, in whom removal of most of the adrenal tissue resulted in the cure of both conditions, has been reported by Stanford and Greene (1970). These authors suggested that premenstrual and menstrual migraine may be caused by an excessive secretion of aldosterone. In order to further investigate this hypothesis, it would be necessary to measure the aldosterone secretion rates in normal controls and in women suffering from menstrual migraine. One fact which would appear difficult to reconcile with this hypothesis is that aldosterone secretion increases in pregnancy (Vande Wiele et al., 1960), a condition usually associated with an improvement in migraine.

The Role of Prostaglandins in Migraine

The prostaglandins are a group of long chain fatty acids with one or more free unesterified alcoholic OH groups which are essential to their biological activity. First isolated from seminal fluid by von Euler in 1934, they have been isolated subsequently from many tissues (Bergstrom, 1967) There are 6 "primary" prostaglandins which differ from eachother by the position and number of -OH groups, and the presence or absence of one or two cis double bonds.

These 6 "primary" prostaglandins fall into two groups according to their physiological actions. Each group consists

of 3 members, named thus:

Prostaglandins E: PGE_1, PGE_2 and PGE_3

Prostaglandins F: $PGF_{1\alpha}$, $PGF_{2\alpha}$ and $PGF_{3\alpha}$

The prostaglandins are receiving intense study at the present time, and it appears that these substances have many important physiological actions. The most relevant of these to the present discussion are the role of prostaglandins in menstruation, their effects on vascular smooth muscle, their serotonin-releasing properties, and their ability to precipitate migraine.

Prostaglandins in menstruation

Two prostaglandins which have an oxytocic action, $PGF_{2\alpha}$ and PGE_2 have been isolated from menstrual fluid by Clitheroe and Pickles (1961). The menstrual prostaglandins are probably formed in the endometrium during menstruation, since the amount of prostaglandins dischanged considerably exceeded the amount estimated to be present in the endometrium (Eglington, Raphael, Smith, Hall and Pickles, 1963), and since it has been shown that endometrial curettings are capable of synthesising prostaglandins in vitro (van Dorp, 1966). The prostaglandin content of menstrual fluid is reduced in anovulatory cycles (Pickles, 1966), and therefore is probably increased by progesterone

secretion. There is some evidence for the presence of a circulating oxytocic lipid, possibly a prostaglandin, during menstruation (Pickles, 1959).

The action of prostaglandins on vascular smooth muscle

Prostaglandins of the E and A series are potent vasodilators in most vascular beds, whereas Prostaglandins F have only a weak vasodilatory action on arterioles. This topic has been reviewed by Horton (1969).

Prostaglandin-induced serotonin release

The similarity in the action of serotonin and prostaglandins on smooth muscle led Thompson and Angulo (1969) to study the release of serotonin from tissues by prostaglandins in the rat. They found that prostaglandin PGE_1 produced significant depletion in the tissue stores of serotonin in various organs.

Precipitation of Migraine by Prostaglandins

In studying the clinical and metabolic effects of an intravenous infusion of PGE_1 in 8 healthy, non-migrainous males, Carlson, Ekelund and Oro (1968) reported that a throbbing vascular headache developed in all subjects during the infusion. One of their volunteers experienced a severe, unilateral headache which was accompanied by visual symptoms of flashes of light, resembling migraine. These

workers commented that the other changes produced by the intravenous infusion of PGE_1 resembled those produced by intravenous infusion of serotonin. These changes included facial flushing and elevation in plasma levels of free fatty acids (FFA).

In view of these findings, it is possible that the fall in plasma serotonin which usually accompanies the migraine attack, and the unilateral, severe throbbing headache of migraine, may be caused by the release of prostaglandins (e.g. PGE_1) into the circulation. This postulated release of prostaglandins may in turn be caused by the withdrawal of oestradiol or its metabolites during the premenstrual phase. Thus, prostaglandin release, triggered by oestradiol withdrawal, may be responsible for menstruation, and, in constitutionally-predisposed women, may lead to migraine.

At this stage, it will be necessary to recapitulate briefly the main facts established in this study.

It has been shown that premenstrual and menstrual migraine occur during, or at the termination of, the phase of falling plasma levels of both oestradiol and progesterone. Treatment of migrainous women with progesterone in order to prevent the normal physiological decline in plasma progesterone levels was found to have no prophylactic value against

migraine in the majority of women. Furthermore, it was shown that withdrawal of progesterone following the cessation of treatment did not provoke migraine in any of the women studied.

Treatment of migrainous women with depot injections of oestradiol valerate during the premenstrual phase succeeded in postponing migraine by 3 to 9 days in each subject. It was found that the delayed migraine began after plasma levels of oestradiol had fallen to low values. In the one woman who was given a second injection of oestradiol 6 days later in an attempt to further postpone the headache, the migraine occurred during a phase of increasing plasma oestradiol. In her case, it appeared that the initial withdrawal of oestradiol had set in motion a series of events which were no longer responsive to the plasma concentration of oestradiol. This raised the possibility that migraine may be caused by the withdrawal of a metabolite of oestradiol, rather than by the withdrawal of the principal hormone itself.

A typical migraine attack followed the injection of oestradiol in two amenorrhoeic women who had been previously subject to menstrual migraine. In each case, the migraine began after plasma levels of oestradiol had fallen nearly to baseline levels, 10 to 11 days after the initial injection.

Since these women were shown to have consistently low plasma levels of progesterone, it was considered that migraine in these women must have been caused by the withdrawal of oestradiol.

Measurement of plasma levels of serotonin once daily during the premenstrual and menstrual phases of the cycle in groups of normal, non-migrainous women, and women subject to regular menstrual migraine failed to show any significant difference in mean levels of plasma serotonin around the time of menstruation, which might account for the clustering of migraine attacks around this time.

A pilot trial involving the continuous daily administrat--ion of a progestogenic hormone, 3-acetoxychlormadinone (AY 11440) in the attempted prophylaxis of migraine, failed to show any convincing benefit attributable to the drug. It was noted, however, that many women experienced slight breakthrough bleeding, often associated with migraine. It was felt that a likely cause for the simultaneous occurrence of breakthrough bleeding and migraine was fluctuation in oestrogen levels.

The clinical study of 200 women during the last 4 weeks of pregnancy confirmed the orthodox view that pregnancy usually exerts a beneficial effect upon migraine. In a small

proportion of women, migraine began during pregnanc, often during the first trimester. Mean plasma levels of progesterone, measured in normal pregnant women, in migrainous women who had improved during pregnancy, and in migrainous women who had failed to improve during pregnancy, showed no significant differences between groups, suggesting that some factor other than a "protective" effect of progesterone, may be responsible for the beneficial effect of pregnancy on migraine. Some of the possible mechanisms involved have been considered.

Before advancing a hypothesis on the role of female hormonal changes in the aetiology of migraine, the known actions of oestradiol and progesterone on the vascular system will be discussed.

The Actions of Oestradiol and Progesterone on Uterine Blood Vessels

The classical work of Markee on the mechanism of menstruation has been described above. Confirmation of his findings has been provided by Salvatore (1968), who carried out a careful histological study of endometrial biopsies of normal women during different phases of the cycle. He found that during the secretory phase, there is a dilatation of the spiral arterioles (enlargement of the

lumen as well as hypertrophy of the walls), and that, in the immediate premenstrual phase, a pronounced constriction in these arterioles appears. During menstruation, there is a prominent, widespread vasoconstriction affecting many segments of these arterioles.

Uterine bleeding may follow the withdrawal of oestrogen alone, or the withdrawal of progesterone after a period of "priming" with oestrogen (Corner, 1951). Thus, the process of uterine bleeding requires some prior exposure of the uterine vessels to oestrogen, since progesterone withdrawal alone does not initiate menstrual bleeding.

The Actions of Oestradiol and Progesterone on Systemic Blood Vessels

Surprisingly little data has been accumulated regarding the effect of female sex hormones on systemic blood vessels. Hagen (1922) noted premenstrual vasoconstriction in the nailfold capillaries. Landesman, Douglas, Dreishpoon and Holze (1953) studied the circulation of the bulbar conjunctiva through the slit lamp microscope, and found that menstruation, and in some cases, ovulation were associated with constriction of arterioles. They found that from day 3 to ovulation (i.e., the proliferative, or oestrogen-dominated phase), there was a progressive increase

in vascularity and blood flow. Vasoconstriction appeared immediately before menstruation. Using digital plethysmo--graphy, Oppo and Albertazzi (1962) demonstrated arteriolar spasm during menstruation, compared to the intermenstrual interval. McCausland et al. (1963) demonstrated that venous distensibility measured in digital vessels closely followed the progesterone curve, and was greatest one week pre-menstrually. The hypotensive effect of large doses of progesterone reported by De Soldati and Suarez-Navas (1962) has been mentioned previously.

These findings appear to indicate that widespread arteriolar constriction, affecting uterine and systemic vessels, occurs during the premenstrual and menstrual phases, as a consequence of withdrawal of oestradiol and progesterone, and that progesterone has an inhibitory effect on vascular smooth muscle. Whether oestradiol increases vascular reactivity is a question which has not yet been adequately investigated.

Vasomotor Reflexes and Migraine

Attempts have been made to demonstrate some abnormality in the vasomotor reflexes in migrainous subjects. Appenzeller (1963) studied the response in forearm blood flow to radiant heating of the trunk, and found this reflex

to be defective in 8 out of 10 migrainous subjects. His results have been challenged on the basis that treatment with ergotamine could have caused this effect. On the contrary, Elkind, Friedman and Grossman (1964) found no difference in resting forearm skin blood flow between migrain--ous and normal persons, either during the attack, or in headache-free periods. Similarly, Hockaday, Macmillan and Whitty (1967) could find no impairment of these reflexes in migrainous women, irrespective of their stage of the menstrual cycle, or whether or not they were taking oral contraceptives.

Migraine and Development of the Endometrial Arterioles

Recently, Grant (1965 and 1968) has drawn attention to the high incidence of well developed spiral arterioles of the endometrium, as discovered by examination of endometrial biopsies, in women suffering from menstrual migraine. She distinguished a "headache susceptible" or "vascular reactive" group of women from the appearance of the arterioles in the biopsy specimen. She found that the incidence of headaches during the first year on oestrogen-progestogen treatment correlated closely with the incidence of well developed endometrial arterioles. Although her work remains unconfirmed at present, it is tempting to speculate that similar arteriolar hypertrophy

under the influence of the synthetic hormones, may occur elsewhere in the body, including the scalp vessels, and that these abnormally hypertrophied vessels might respond to the hormonal changes, leading to migraine in susceptible women.

Grant found some correlation between the degree of arteriolar development and the dose of progestogen used. It is possible that the vascular hypertrophy she observed was in fact the result of oestrogen stimulation, since all of the progestogenic compounds in her study were of the C_{19}-nor variety, which are metabolised to a significant extent to oestrogenic compounds.

X

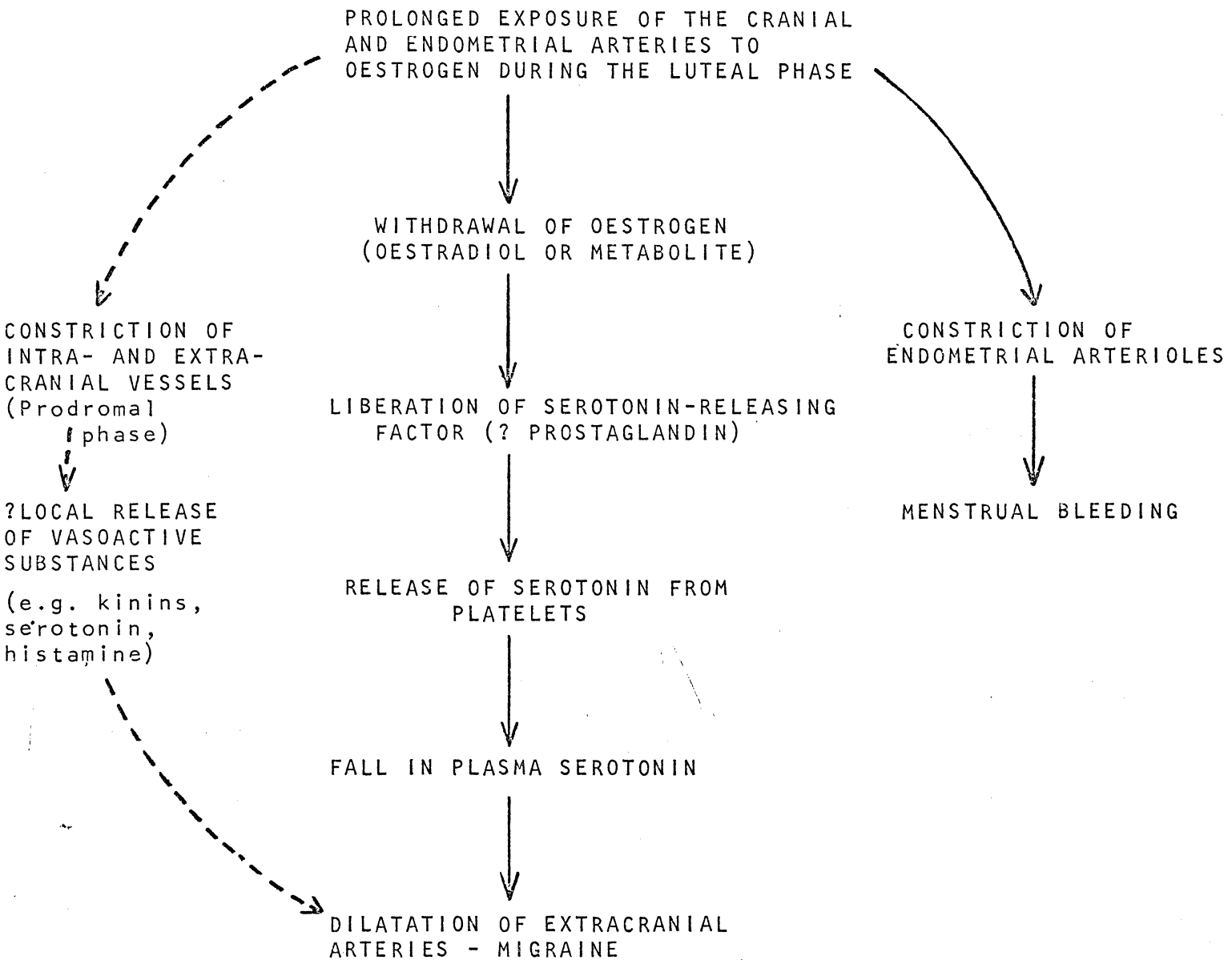
HYPOTHESIS

A hypothesis concerning the mechanism whereby changes in female sex hormones may precipitate migraine is shown in figure 12.1.

The withdrawal of oestradiol and its metabolites from the circulation during the premenstrual phase results in widespread arteriolar dilatation. Oestradiol withdrawal also results in the liberation of a serotonin releasing factor (SRF) which is possibly a prostaglandin. SRF then releases serotonin from platelets, leading to a decrease in plasma levels of serotonin, and a rise in the urinary excretion of 5-HIAA, and the precipitation of the migraine attack. SRF may also be involved in the mechanism of menstrual bleeding.

It should be mentioned that only prolonged exposure of the cranial vessels to oestrogen appears to be effective in precipitating migraine. Thus, the rapid rise and fall of oestradiol during the preovulatory peak do not seem to be capable of precipitating migraine, although in exceptional cases, migraine may recur at mid-cycle, with or without menstrual migraine.

Figure 12.1 - Hypothesis on the Action of Oestrogen Withdrawal in Migraine



SUGGESTED LINES OF FUTURE RESEARCH

Several aspects of the current work appear to offer themselves as topics for fruitful research. These include:

1. Investigation of the possible role of oestradiol metabolites in the pathogenesis of menstrual migraine. This could be conducted along lines similar to those used for the study of the effect of oestradiol on migraine described in the present study. As a preliminary step, the role of oestrone withdrawal could be studied.

2. Study of the effect of oestrogens and progesterone upon vascular smooth muscle, particularly the media of the scalp arteries, in normal and migrainous subjects. In vitro experiments involving a study of the responses of a segment of superficial temporal artery to various vasoactive substances may provide information concerning the effects of these hormones if such arterial segments were obtained from women migraine sufferers at various phases of the menstrual cycle.

3. Further investigations of the role of prostaglandins in migraine, with particular reference to a study of the ability of these compounds to release serotonin

from platelets, and the possible role of prostaglandins in the mechanism of menstruation.

4. A clinical study of the effect upon migraine of continuous administration of oestradiol to women subject to menstrual migraine, in a dose sufficient to provide a constant plasma level of the hormone, while suppressing endogenous oestradiol secretion.

5. Serial measurement of plasma levels of progesterone and oestradiol during pregnancy in normal women, in women migraine sufferers who have obtained relief during pregnancy, and in migrainous women who have failed to experience relief during pregnancy, in order to see whether failure to obtain relief from migraine could be related to exaggerated fluctuations in the level of either hormone.

6. A survey of the incidence of migraine in women taking oestrogen-progestogen combinations, to ascertain whether the incidence of migraine can be related to the dose of synthetic oestrogen rather than the dose of synthetic progestogen.

SUMMARY OF THE ORIGINAL CONTRIBUTIONS MADE BY THIS THESIS

This thesis comprises principally a biochemical, and to a lesser extent, clinical study of the importance of changes in the levels of circulating progesterone and oestradiol in the aetiology of migraine. The original contributions arising from this work may be summarised as follows:

1. A study of the normal pattern of plasma levels of estradiol and progesterone by serial daily blood sampling throughout the menstrual cycle, has been made in a number of normal women. The pattern of plasma levels of these hormones had not hitherto received such detailed study, and basic knowledge concerning ovarian physiology, particularly regarding the corpus luteum of menstruation and early pregnancy, has been gathered.

2. Daily plasma concentrations of progesterone and oestradiol have been studied throughout the menstrual cycle in women subject to regular, predictable premenstrual or menstrual migraine. It was found that migraine occurred during or just after the phase of simultaneous withdrawal of progesterone and oestradiol.

3. Using an experimental approach, the individual contribution of progesterone withdrawal to the precipit--ation of menstrual migraine has been studied. It was found that progesterone does not appear to have a a protective effect against menstrual migraine, and that the withdrawal of progesterone alone does not precipitate menstrual migraine. This latter finding is contrary to the hypotheses of previous workers.

4. Similar experiments involving treatment of migrainous women with oestradiol revealed that the injection of oestradiol valerate during the premenstrual phase invariably postpones migraine, and that migraine occurs following the withdrawal of oestradiol (in this case, by the metabolic clearance of the injected hormone).

5. A clinical study of 200 pregnant women has shown that, in accord with earlier views, pregnancy usually exerts a beneficial effect upon migraine; however, in partial confirmation of recent work by Callaghan (1968), it was found that, in a small proportion of women, migraine may begin during pregnancy, most commonly in the first trimester.

6. A biochemical study combined with the clinical survey of migraine in pregnancy failed to show any significant difference in mean plasma concentrations of progesterone between groups of normal, non-migrainous pregnant women, women whose migraine had remitted during pregnancy, and women who had failed to experience remission, when assays were performed on single blood samples obtained in the 4 weeks before the expected date of confinement.

7. A pilot trial of a synthetic progestogenic hormone, 3-acetoxychlormadinone (AY 11440) failed to show any significant protective effect against migraine. Clinical observations made during this trial suggested a link between fluctuations in plasma levels of oestrogens and migraine.

The implications of these findings have been discussed, and an attempt has been made to unify them into a single hypothesis regarding the role of fluctuations in plasma concentrations of oestradiol and progesterone in the aetiology of migraine.

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STATISTICAL APPENDIX

Statistical analysis of the data obtained was carried out by Dr. R. Vagholker, Department of Statistics, School of Mathematics, University of New South Wales. The results are summarised below.

1. Comparison of oestradiol assay results obtained in two different laboratories (p.70):

Analysis of variance table:

Source of variation	D.F.	S.S.	M.S.	F
Location	1	0.7004	0.7004	1
Days	1	266.0004	266.0004	83.34^{**}
Patients	5	219.0737	43.8147	13.73^{**}
Location x days	1	0.6337	0.6337	1
Days x patients	5	118.7921	23.7584	7.44^{**}
Location x patients	5	44.8371	8.9674	2.81
Error	5	15.9588	3.1918	
Total	23	665.9962		

$F_{0.05,1,5} = 6.61$ $F_{0.05,5,5} = 5.05$

$F_{0.01,1,5} = 16.30$ $F_{0.01,5,5} = 11.0$

Conclusions

(a) The differences in values obtained in the two laboratories are not significant, i.e. the measurement of plasma oestradiol in both laboratories appears to be similar.

(b) There are very significant differences between days (day 7 and day 21) and also between patients.

(c) There is a significant interaction between days and patients. The rate of increase in plasma oestradiol from day 7 to day 21 differs between the patients.

2. Comparison of Progesterone Values with/ without TLC (p.56)

A t-test gave the value of t = -1.21 (not significant).

It was concluded that the difference in values obtained when progesterone was determined using TLC and not using TLC was not significant, i.e., the mean concentration could be assumed to be the same for both methods.

3. Plasma 5-HT levels in normal, non-migrainous women before and during menstruation (p.106)

From the following analysis of variance table, it was concluded that:

(a) The differences in 5-HT levels do not differ significantly from day to day, i.e. the mean 5-HT levels of the group remain the same before and during menstruation.

(b) The differences in 5-HT levels in individual women are very highly significant.

Analysis of variance table:

Source of variation	D.F.	S.S.	M.S.	F
Day	8	0.217364	0.027170	1.95
Women	4	0.425435	0.106359	7.62**
Error	30	0.418725	0.013958	
Total	42	1.061524		

$F_{0.05,8,30} = 2.27$ $F_{0.01,8,30} = 3.17$

$F_{0.05,4,30} = 2.69$ $F_{0.01,4,30} = 4.08$

4. Plasma 5-HT levels in women subject to regular, recurrent menstrual migraine (p.106)

From the following analysis of variation table, it was concluded that:

(a) There are no significant differences in mean 5-HT levels from day to day, i.e., the mean 5-HT levels remain

unaltered significantly during and before menstruation.

(b) The differences in mean plasma 5-HT values between individual women are very highly significant.

Analysis of variance table:

Source of variation	D.F.	S.S.	M.S.	F
Day	8	0.283791	0.035474	1.26
Women	4	2.377742	0.594436	21.08
Error	28	0.789498	0.028196	

$F_{0.05,8,28} = 2.30$

$F_{0.05,4,28} = 2.72$ $\quad F_{0.01,4,28} = 4.08$

5. Plasma Concentrations of Progesterone measured during the four weeks before the expected date of delivery, in:

A. Normal, non-migrainous pregnant women.

B. Migrainous women whose migraine had improved during this pregnancy

C. Women whose migraine had failed to improve during this pregnancy.

Table of Means (Table 11.2, p.122)

Group	Mean level of plasma progesterone (ng/ml)
A	119.38
B	98.41
C	101.23

Analysis of variance table:

Source of variation	D.F.	S.S.	M.S.	F
Between groups	2	3730.66	1865.3	1.45
Within groups	34	43700.80	1285.32	
Total	36	47431.46		

Conclusion: The level of plasma progesterone does not differ significantly from one group to another.