STUDIES ON THE EFFECTS OF DIFFERENT PREPARATIONS OF HUMAN GONADOTROPHINS ON THE EXCRETION OF STEROIDS BY WOMEN

by

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Thesis submitted for the degree of Ph.D.
in the University of Birmingham

OCTOBER 1963

SYNOPSIS

This research work has been a biochemical investigation of the effects of different preparations of human urinary and pituitary gonadotrophins on the excretion of steroids by women with different endocrinological conditions.

The effects of different dosages of hormones were studied in a group of women with secondary amenorrhoea in statistically designed experiments and the results were analysed by comparison to the excretion of steroids during the normal menstrual cycle.

Using the information gained from these experiments these women have become pregnant, and one pregnancy occurred during an experiment. One woman gave birth to dissimilar twins, two to single children and another aborted. The excretion of hormones during pregnancy was followed and compared to the excretion patterns found in normal pregnancy. The excretion of oestriol and pregnanediol following treatment with gonadotrophins and other trophic hormones was studied in a woman with Simmonds' disease.

The hormones controlling the excretion of steroids in the urine of women with the Stein-Leventhal Syndrome and the occurrence of steroids in the follicular fluid from the patients was investigated. During this work a short rapid method for the estimation of oestriol in urine was developed. The accuracy, precision, sensitivity and specificity of the method was discussed.

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INTRODUCTION

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Biological properties of gonadotrophins.

Historical introduction:

The presence (in the anterior lobe of the pituitary gland of hormones possessing specific gonad stimulating effects) was established by the work of Smith & Engle (1927) and Zondek & Aschheim (1927). These workers demonstrated that pituitary extracts induced precocious sexual development in immature male and female rodents. Gonadal stimulating factors were also demonstrated in chorionic tissue (Murata & Adachè, 1928) and in the urine of post-menopausal women and castrated men (Aschheim, 1928; Hamburger, 1931).

Pituitary gonadotrophins:

It was soon clear that pituitary extracts or implants could produce two gonadal reactions (a) the stimulation of follicular growth in the ovaries and of spermatogenic activity in the testis and (b) the final ripening of the ovarian follicles together with the exhibition of oestrus or heat, the rupture of the follicles and their subsequent transformation to corpora lutea, and in the testis the assumption of a functional role on the part of the Leydig cells which secrete testosterone which in turn causes development of the secondary sex glands.

The first separation of pituitary gonadotrophic extracts into two components with their different biological activities was shown by Fevold, Hisaw & Leonard, (1931). The first preparation was called "Follicle stimulating hormone" (F.S.H) and the second "luteinizing hormone" (L.H) or "interstitial cell stimulating hormone" (I.C.S.H). Subsequent work from other laboratories (for review see Evans, 1950), has confirmed the concept that the two hormones F.S.H. and L.H. are present in pituitary extracts. More recently several workers have succeeded in purifying pituitary gonadotrophins; Gemzell, Dicfalusy & Tillinger (1958) by precipitation using ammonium sulphate; Steelman & Segaloff (1959) used a chromatographic procedure that employed ion exchange cellulose; Roos & Gemzell (1960) by chromatography on carboxymethyl and diethylamino-ethyl cellulose followed by zone electrophoresis on a copolymerizate of polyvinyl chloride and polyvinyl acetate. In this laboratory the method of Steelman &

Segaloff (1959) has been modified and extended and F.S.H. has been prepared which is largely free of L.H. (Butt, Crooke & Cunningham, 1961; Butt, Crooke, Cunningham & Wolf, 1963) and in Cambridge L.H. has been obtained which appears to be free of F.S.H. (Hartree, Butt, Kirkham, 1963).

It is now accepted that pituitary extracts contain a third gonadotrophin which in the rat is responsible for the maintenance of activity of corpora lutea and for the secretion of progesterone (Astwood, 1941, 1953; Evans, Simpson, Lyons & Turpeinen, 1941). This substance has been termed luteotrophin (LTH) and may be identical to the lactogenic hormone, prolactin.

Placental gonadotrophins:

Human chorionic gonadotrophin (H.C.G):

The gonadotrophic action of the urine of pregnant women was first shown in normal immature rats and mice in which it induced the formation of haemorrhagic follicles and corpora lutea (Aschheim & Zondek, 1927). When tested in hypophysectomised female rats it induced only stimulation of interstitial cells and did not cause follicular development (Simpson, Li & Evans, 1951). Ovarian hypertrophy is slight (except at high doses) but uterine stimulation due to oestrogen production may be marked. In the male, repair and stimulation of interstitial cells occurs with resulting androgen production. As a result spermatogenic activity may be maintained (Smith & Leonard, 1934; Evans, Penchurz & Simpson, 1934; Simpson et al., 1951). The effects in the intact rat are in sharp contrast to those in the hypophysectomised rat. Thus, in the female injection of H.C.G. is followed by ovarian hypertrophy due to follicular development and corpora lutea. The associated hyperaemia and ovulation in immature animals serve as indices for pregnancy diagnostic tests. In the male the testicular elements are stimulated with resulting hypertrophy of the testis and secondary sex organs. The more commonly accepted explanation of the gametokinetic action of H.C.G. in the male rat is that it is secondary to androgen production by the stimulated Leydig tissue. (The hormone most similar to H.C.G. would be pituitary L.H.).

Human urinary gonadotrophins:

The assumption has been made that because F.S.H. and L.H. activities are readily concentrated in independent

protein moieties from pituitary tissue, the same must be true of urine from non-pregnant individuals. Donini & Marchetti (1953) using chromatography on permutit ion exchange resin obtained a preparation from the urine of post-menopausal women that contained mainly F.S.H. activity. Johnsen (1955) using similar techniques obtained a preparation of greater specific activity. Segaloff & Steelman (1959) also used chromatography on ion exchange resins and presented evidence that it is possible to prepare an L.H. fraction from the urine of non-pregnant individuals, which is remarkably free of F.S.H. At the same time these workers claimed to have isolated urinary F.S.H. in apparently homogenous form. Independently Butt, Crooke & Cunningham (1959) using similar techniques prepared fractions of gonadotrophin from the urine of male subjects which were relatively rich in F.S.H. and L.H. respectively. More recently Bourillon, Got & Marcy (1960) have obtained a preparation of high F.S.H. activity from the urine of post-menopausal women by precipitation with ethanol, absorption on kaolin, chromatography on ion exchange resins and electrophoresis on starch gel.

Pregnant Mare's Serum gonadotrophin (P.M.S).

In 1930 Cole and Hart demonstrated the presence of a potent gonadotrophin in the serum of the pregnant mare which unlike H.C.G. showed physiological activity similar to F.S.H. Biological studies on this gonadotrophic hormone have established additional distinct differences between it and H.C.G., P.M.S. has been found to possess predominantly F.S.H. activity with smaller quantities of L.H. activity (Davis & Koff, 1938; Siegler, 1939). P.M.S. was considered to be a single gonadotrophin (Huber & Davis, 1940) possessing both F.S.H. and L.H. properties in the same molecule until the separation into "F.S.H" and "L.H" fractions by Evans, (1936) and Hellbaum, (1937). However, the preparation of Li, Evans & Wonder (1940) appeared to be electrophoretically homogenous retaining the original physiological property of the serum. Evidence in favour of separate gonadotrophic factors in P.M.S. comes from the work of Kapperman, Meyer & McShan (1941) who produced an anti-serum against sheep pituitary F.S.H. which inhibited the follicle stimulating factor of the injected mare serum without affecting the action of the luteinizing factor.

From the original report by Cole & Hart, (1930) of the potent gonadotrophic effects of P.M.S. on the ovaries of rats, further extension of the work showed that it had the effect not only of inducing follicular development ovulation and corpus luteum formation in hypophysectomised animals, (Palmer, 1957) but also in intact mice, ewes, sows, cows and rabbits (Catchpole & Lyons, 1934; Van Wagenen & Cole, 1938; Siegler, 1939, and Kurland, 1951).

Clinical Trials with Gonadotrophins:

Attempts to induce ovulation.

The effects of human chorionic gonadotrophin on ovulation.

Many investigators have used H.C.G. in an attempt to stimulate the ovaries of non-pregnant primates. The ready availability of this source of gonadotrophin, the development of preparations without general or local reactions and the early misconception that the substance was of pituitary origin served to stimulate extensive investigation and therapeutic trials. Early in the investigations some workers reported stimulation of ovulation and corpus luteum formation in women (Novak & Hurd, 1931, and Kenny & Daley, 1940). Reports have continued to appear during the past 25 years claiming that H.C.G. may cause ovulation, however, the results of Engle (1933), Hartman, (1934), Marshall, (1935) and Johnson, (1935) with the monkey as well as the results of Giest, (1933), Westman, (1935), Hamblen, (1939)(1940), Brown, Bradbury & Metzger, (1941), and Bushnell, (1948) in women demonstrate clearly that H.C.G. does not stimulate the development of Graafian follicles or cause maturation and liberation of ova in primates. Pregnancy when it occurred was only coincidental (Palmer, 1957). Instead of causing secretion of oestrogen and ovulation there is evidence that the large follicles became atretic while smaller follicles were hyalinised, concomitant with decreased ovarian secretion (David, 1947). Brown & Venning (1938) demonstrated that prolonged treatment with H.C.G. in women having normal menstrual cycles leads to atrophy of the endometrium which suggested suppression of oestrogen production by the ovaries.

In Scandinavia, on the other hand, interest in the therapeutic uses of H.C.G. has continued and reports of its successful use have continued to appear. These workers, including Rydberg, (1943); Pahlson, (1951),

Wahlen, (1952), Bergman, (1958), have shown that, in patients with metropathia haemorrhagica cystica, a high success rate is possible but the authors stipulated that before H.C.G. could be effective, the ovaries must contain more or less mature follicles as they do in metropathia haemorrhagica cystica.

The effect of pregnant mare's serum (P.M.S) on ovulation.

Initially studies on the therapeutic uses of the substance were retarded because of the technical difficulties involved in the isolation of this gonadotrophic activity from blood. Cartland & Nelson (1937, 1938) obtained a preparation largely free of serum proteins which they standardised by measuring the increase in weight of the ovaries of rats. Since then many experimental trials of the effects of P.M.S. in women were undertaken and evidence of luteinization in the ovaries was obtained by Buttner, (1937); Watson, Smith & Kurzrok (1938). However, when P.M.S. was given to women in whom the ovaries were later removed (Westman, 1943) evidence was obtained of follicular development but there were no signs of corpora lutea formation. Histological evidence showed that the preparation caused ovulation and follicular development in 16 of the 30 women treated by Siegler & Fein, (1939), Siegler, (1940). The clinical use of P.M.S. was found to be inconsistent and disappointing. Despite the evidence that it produced follicular stimulation, ovulation followed by corpus luteum formation rarely occurred (Hamblen, 1938; Giest, Gaines & Salmon, 1941, and Rydberg, 1943).

The effect of the combinations of H.C.G. and anterior pituitary extract (A.P.E.) or P.M.S. on ovulation.

H.C.G. has been widely used clinically as an ovulatory factor. To be effective it was administered after maturation of the follicles was thought to have occurred under the stimulating effect of extracts of the anterior pituitary or of pregnant mare's serum. The effectiveness of such a combination of treatment has been proved in laboratory animals (Engle & Hamburger, 1935) and it was found that the administration of H.C.G. with anterior pituitary extracts greatly enhanced the action of the latter in rodents (Leonard, 1932; Mazer & Katz, 1933; Bodemer, Rumery, & Blandau, 1959). Mazer & Ravetz (1941) studied the effect of H.C.G. together with animal anterior pituitary extract Synapoidin (Parke Davis) when given to 23 women over a period of 1 - 18

days before laparotomy. In 20 of the 23 women there was definite evidence of ovarian stimulation and microscopic examination of the ovaries of 16 of these women revealed in all but one of the women, multiple haemorrhagic follicles with luteinized granulosa layers. Two of the patients who were considered to have anovulatory cycles conceived and periods followed in 19 of the 23 women. In some of the women with normal ovaries treatment caused over stimulation as judged by an increase in size at laparotomy.

From these studies the idea originated that better results could be obtained by injecting H.C.G. after the follicles had been stimulated than by the simultaneous injection of H.C.G. and F.S.H. The use of P.M.S. to stimulate follicular development, followed by H.C.G. to induce ovulation and luteinization appeared a logical approach and has been used by Rydberg & Pedersen-Bjergaard, (1943); Hamblen & Davis, (1945); Riisfeldt (1949) with greater success than has treatment with the hormone alone. The results of Wahlen (1952) indicated that in the presence of a mature follicle, ovulation could be induced by the administration of H.C.G. alone. Igorashi & Matsumoto (1957) used P.M.S. followed by H.C.G. but they used individual doses of hormones for different patients as suggested from a daily examination of cervical mucus which indicated in an empirical way the state of maturity of the follicle. H.C.G. was injected when the follicle was thought to be mature. Recently a number of deaths following intravenous Synapoidin therapy have been encountered in America so that this approach can no longer be justified (Swyer, 1963).

The effect of human pituitary gonadotrophin on ovulation.

Gemzell et al (1958) were the first workers to successfully use human pituitary F.S.H. (HP-FSH) to induce ovulation in women with amenorrhoea. These workers used a partially purified material in a daily dosgae of 10mg. equivalents of HMG-IRP, as judged by the ovarian augmentation assay in rats, for 10 days followed by H.C.G. 6,000 i.u./day for 4 days. The effects of the treatment were followed by measuring the excretion of oestrogen and pregnanediol in urine and by recording the body temperature and by the examination of vaginal smears. The ovaries of some of the patients treated were also examined by culdoscopy. There was evidence of ovulation in 29 out of a group of 40 women. Some of

these women had secondary amenorrhoea while others had primary amenorrhoea with gonadal dysgenesis. In some instances the responses in oestrogen and pregnanediol were enormous and polycystic enlargement of the ovaries was observed at laparotomy. These responses suggested that the doses of gonadotrophin used was too high and this has since been confirmed by the large number of multiple pregnancies which followed treatment (Gemzell, 1962).

It was found that repeated ovulations could be obtained in the same women using pituitary preparations in contrast to the finding that P.M.S. induced anti-hormone formation (Maddock, 1949). Later work has shown that the effect of exogenous gonadotrophin is abolished by the simultaneous administration of progesterone (Gemzell, 1960) and testosterone propionate (Diczfalusy, 1962). These authors have suggested that these two compounds exert their effects at the ovarian level and render the ovary insensitive to gonadotrophic stimulation. Neither were (Gemzell et al, 1958) able to induce any ovarian changes to gonadotrophin in 4 women that were pregnant, the lack of response has been attributed to the high circulating levels of blood progesterone. The same authors attempted to shorten the successive treatments to periods of less than one month but no responses were obtained since the level of blood progesterone was elevated as a result of corpus luteum formation during the first month of treatment. These findings suggest that inhibition to gonadotrophin can exist at ovarian level.

The finding of Gemzell (1961) that higher levels of HP-FSH were effective in inducing ovulation was based on the results of treatment ofahypopituitary dwarf with HP-FSH 1mg, 2mg. 3mg. 5mg. 10mg./day for 10days. The only response obtained was with a dose of 10mg. and this has been used unchanged for the treatment of all patients with amenorrhoea. H.C.G. was found to induce ovulation in 2 out of a group of 50 amenorrhoeic women but the effects of the dose of HP-FSH used in conjunction with the dose of H.C.G. was not investigated.

Buxton & Hermann (1961) administered a daily dose of 20mg./day for four days of a human pituitary F.S.H. preparation to women with amenorrhoea and found evidence for ovulation in 5 of the 7, as judged by changes in body temperature, endometrial biopsy and vaginal smears. Ovulation occurred when H.C.G.

(16,000 i.u./day) for 4 days) was given after F.S.H. but not when the two hormones were used simultaneously. Human pituitary F.S.H. followed by H.C.G. (6,000 i.u/day for 6 days) has been used by Rosemberg, Coleman, Gibree, Mac Gillivray,(1962) to induce ovulation in two women with secondary amenorrhoea. With the low doses of F.S.H. used by Rosemberg et al (1962) the responses obtained were small as shown by excretion of oestrogen and pregnanediol but with larger doses of F.S.H. (10mg/day for 5 days) followed by 5mg./day for /5 days together with 6,000 i.u. H.C.G/day, the same type of enormous response as frequently occurred in some of the patients described by Gemzell et al (1958), Gemzell (1961) was obtained.

The effect of Human Urinary gonadotrophin on ovulation.

The gonadotrophic material prepared from the urine of post-menopausal women (Pergonal) has been used successfully to induce ovulation. Lunenfeld, Rabau Rumney & Winkelsberg (1961) used Pergonal (13.6mg./day) for periods between 8 - 14 days in two women with secondary amenorrhoea. The last doses of Pergonal were given simultaneously with H.C.G. (6,000 i.u./day) for periods between 5 - 10 days and the changes in steroid excretion and body temperature charts suggested that ovulation had taken place. Pregnancy occurred in two women during ovulatory cycles induced by the combined Pergonal-H.C.G. treatment (Lunenfeld et al, 1962). The patients became pregnant during the first and fifth months of treatment.

Six women with secondary amenorrhoea have also been treated with Pergonal by Rosemberg et al (1963). The doses used ranged from 6.8 to 27.2mg./day over periods of 5 - 15 days. H.C.G. (6,000 i.u./day) for 5 days was used together with the last injections of Pergonal. Changes were observed in the oestrogen and pregnanediol levels, and these in conjunction with the changes in body temperature and endometrium suggested that ovulation followed treatment. No pregnancy occurred after any of the induced cycles. Shelesnyak (1962) has reported pregnancy occurring in 2 of 4 patients treated with human pituitary F.S.H. in a series of experiments of factorial design but the scheme of treatment or the method of assessment of ovarian responses has not been published.

Introduction to Section on the treatment of women with the Stein-Leventhal Syndrome.

The Stein-Leventhal Syndrome (1935) is characterised by oligomenorrhoea or amenorrhoea, sterility and enlarged polycystic ovaries and frequently by hirsutism of a masculine type. Originally Stein and Leventhal (1935) proposed that the syndrome was due to an increased production of pituitary gonadothrophins which resulted in the stimulation of a large number of ovarian follicles. However, this thery has not been established. Many other theories have been suggested to explain the syndrome and histological studies, estimations of steroids in urine and follicular fluid, and studies involving incubation of ovarian tissue slices and homogenates from patients with the syndrome have been made.

The metabolic pathways for the formation of oestrogen in the normal human ovary according to Ryan & Smith (1961, a,b) and Smith & Ryan (1961) are shown diagramatically.

Short & London (1961) and Short (1962) have suggested that the ovaries of patients with the Stein-Leventhal Syndrome do not synthesise oestrogen due to the failure of the enzyme responsible for 19-hydroxylation. This leads to an accumulation of androstenedione which has been found in high concentration in the ovarian cyst fluid of these patients. Axelrod & Goldzieher (1961) where unable to detect any oestrogen when ovarian slices from patients with the Stein-Leventhal Syndrome were incubated with various steroid substrates. They concluded that their negative findings resulted from a blockage in the conversion of 19-hydroxy androstenedione to oestrone. A similar approach was adopted by Mahesh & Greenblatt (1961; 1962) who found large quantities of Δ 5-compounds in the follicular fluid and urine of these patients. The Δ 5-compounds consisted of dehydroepiandrosterone (DHA), 17-αhydroxy Δ 5-pregnenolone and Δ 5-pregnenetriol.

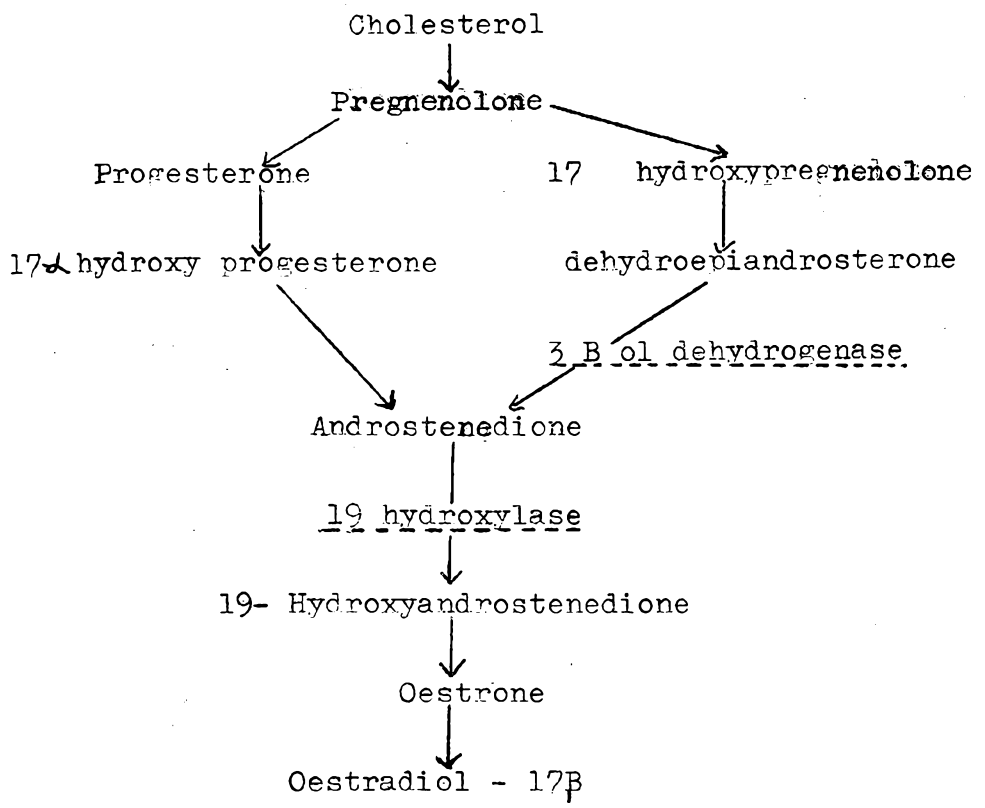


Diagram of 2 of the biosynthetic pathways leading to the formation of oestrogen by the human ovary. The two enzymes that are considered deficient in the ovaries of women with the Stein-Leventhal Syndrome are also shown.

They proposed, therefore, that the enzyme defects occurred in the 3β - ol dehydrogenase system that converts the △ 5-compounds into androstenedione. This matabolic pathway is well established in the ovary and the proposed enzyme defect is shown in figure. Both theories explain the failure of such ovaries to produce oestrogen but apparently conflict in the location of the enzyme defect.

Several workers have investigated the effects of exogenous gonadotrophin in patients with the Stein-Leventhal Syndrome. Keetal, Bradbury & Stoddard (1957) used log F.S.H. and showed that the ovaries of 11 of 12 patients treated were greatly enlarged following treatment. Increased levels of urinary DHA and 17-ketosteroids were frequently observed. Similar findings were reported by Borell (1954), Pensonen, Timonen & Mikkonen (1959) using H.C.G. and Gemzell et al (1959; 1961) also noted a marked increase in 17-ketosteroids and 17 hydroxycorticosteroids in patients treated with

human F.S.H and H.C.G. Mahesh & Greenblatt (1961) used a preparation of human pituitary F.S.H. and by giving dexamethasone at the same time suppressed the production of adrenal cortical steroids. They found increased levels of DHA, pregnenetriol, pregnanetriol, 17α hydroxy pregnenolone, etiocholanolone and androsterone in the urine after treatment with F.S.H. and at laparotomy the ovaries of the woman were greatly enlarged and typical of the syndrome. The excretion of oestrogen was not measured in this patient. Dexamethasone was used by Netter, Jayles, Musset, Lambert, Mauvais-Jarvis (1960) who found that increased levels of oestriol, 17-ketosteroids and androsterone occurred after treatment with H.C.G. (10,000 i.u./day for 2 days), little change was noted in the other oestrogen levels and to explain these findings the authors concluded that the enzyme defect in the ovaries of the patients treated lay in an exaggerated excretion of 16-hydroxylmetabolites such as oestriol and 16-hydroxyoestrone. More recently using dexamethasone Bailieu, Mauvais-Jarvis & Corpechot (1963) have shown that increased levels of androsterone and aetiocholanolone were excreted after treatment with H.C.G. (5,000 i.u./day for 3 days) and both androstenedione and D.H.A. were tentatively identified in homogenates of the ovarian tissue removed at wedge resection.

In this work the effects of treatment with F.S.H. and with F.S.H. and H.C.G. were studied in an attempt to show differences in the production of steroids. Dexamethasone was used in the first three patients in order to suppress the production of adrenocortical steroids so that it could reasonably be assumed that any steroids formed after treatment with gonadotrophin were specifically of ovarian origin. Follicular fluid was collected from all patients treated and the steroids present were estimated. The cyst fluid was collected at laparotomy which was performed at different times during and after treatment so that a comparison could be made between the excretion of urinary steroids and the steroids found in the follicular fluid. By this means it was hoped to establish that treatment with F.S.H. and with F.S.H. and H.C.G. could induce changes in the constituents of the cyst fluid. The effects of gonadotrophin on the excretion of D.H.A, androstenol, 17-oxosteroids and 17-oxogenic steroids were also investigated. In addition the effect of gonadotrophin

on oestriol and pregnanediol excretion in urine and progesterone and oestrogen formation in the follicular fluid was studied.

Objectives of the present work.

The main objective of the present work was to obtain information about the effect of human urinary and pituitary gonadotrophin on the ovary as judged by the excretion of steroids in urine.

The method of Brown (1955) appeared to be a convenient method for the estimations of oestrogen. The normal pattern for the daily excretion of oestrone, oestradiol and oestriol has been reported (Brown, 1959). Oestriol is excreted in the greatest quantity and fluctuates by the greatest amount during the normal menstrual cycle and was therefore considered a suitable estimation of ovarian activity. The method of Brown (1955) for the estimation of oestriol is lengthy for routine use and one of the objectives was to simplify the method without sacrificing specificity and sensitivity. The method of Klopper, Michie & Brown, (1955) has been widely adopted for the estimations of pregnanediol and the normal pattern of excretion has been published (Klopper, 1957).

It was decided to investigate three different kinds of clinical treatment; (i) the treatment of women with secondary amenorrhoea. The purpose of this study was to compare the effectiveness of pituitary and urinary F.S.H. preparations of various degrees of purity, given in combination with H.C.G. A series of experiments of factional design were used to establish the optimum conditions for their administration of the hormones with the ultimate object of inducing ovulation and enabling the women to become pregnant. (ii) A study was performed in a woman with Simmonds' disease so that the effects of the treatment were uncomplicated by endogenous hormones.

Finally the study of women with the Stein-Leventhal Syndrome: The recognised treatment for this syndrome is wedge resection of the ovaries and this afforded the opportunity of studying both the steroids obtained from ovarian cyst fluid and urine.

INTRODUCTION TO CLINICAL TRIAL.

The Patients.

The patients selected had complained of infertility associated with secondary amenorrhoea of unknown aetiology of at least three years duration. They were chosen because they were considered to be sufficiently intelligetnt, co-operative and willing to undertake a series of trials with human gonadotrophins. They had no other complaints and their excretion of urinary gonadotrophins was in the lower half of the normal range on at least three occasions.

They were coded alphabetically in the order in which they volunteered for treatment, with the intention that if one gave up for any reason whatever, she would be replaced by the patient having the next letter.

They were admitted to the ward for clinical investigation which included a pelvic examination under anaesthesia, uterine curettage, tubal insufflation and culdoscopy. All other investigations including the chromosonal pattern of buccal or vaginal smears gave normal results. The husbands' semen specimens were also normal. Table IAshows that all patients had small uteri and the endometrium, when obtainable, was scanty and inactive. Culdoscopy revealed small ovaries with glistening white capsules and no cysts or only occasional tiny subcapsular translucent cysts visible.

The Objective of the Experiement.

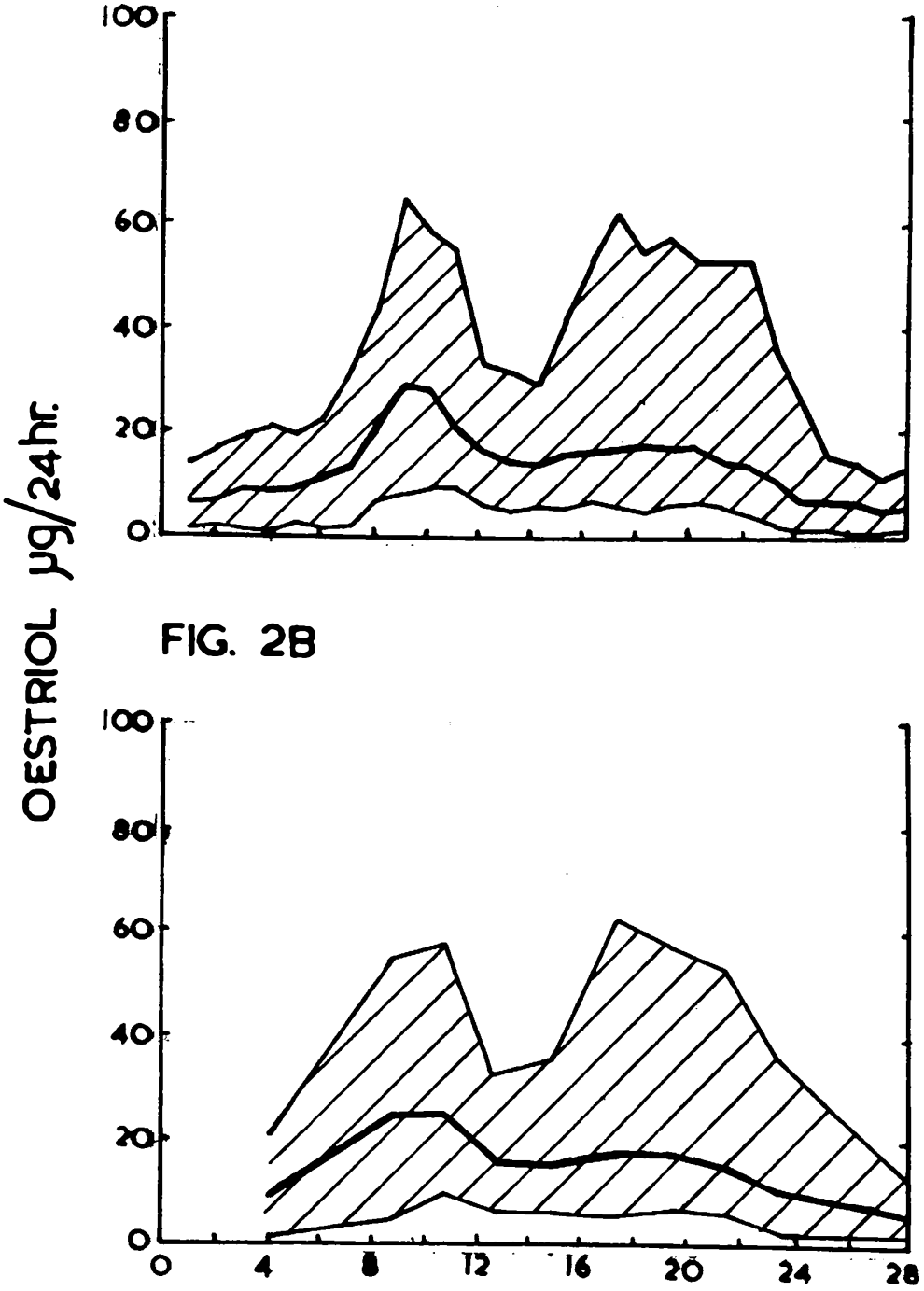
The normal pattern of daily oestriol excretion was reported by (Brown, (1959). It was based on observations on sixteen women aged 18 to 41 years who had regular menstrual cycles. The mean, maximum and minimum figures are shown in Fig. 2A. From this figure it was seen that the oestriol level increased to a maximum at about day 10 and remained at an elevated level for about 4 days after which it fell on about day 14. In some cases it rose again to form a secondary oestriol peak and in some of the women the secondary rise (luteal phase) was higher than the primary one (ovulatory phase). Some of the women showed no secondary rise so that apparently it is not a requisite for ovulation. The oestriol always had fallen after the secondary rise before menstruation occurred.

Table I.

Patients	A	B	C	D	E
Age	34½	32	23½	25½	27
Menarche (age)	13-14	16	15	13½	11
Cata	3/28	5/28	6/28	5/30	4/28
Cessation	Gradual	Gradual	Abrupt	Abrupt	Abrupt
Last menstrual period (age)	27	29	20	22	20
Ovaries size (cm)	1.5x0.5	1.5x0.5	2.0x0.5	1.0x1.0	2.5x1.2
Ovaries, wall	Thin	Thin	Thin	Thin	Thin
Ovaries, cysts	None	None	One small	One small	Two small
Tubes	Patent	Patent	Patent	Patent	Not examined
Uterus, sound (cm)	6.25	5.0	6.25	5.0	5.0
Ratio cervix-body	Ratio 1/1	Ratio 1/1	Ratio 1/1	Ratio 1/1	Ratio 1/1
Uterus, endometrium	Nil	Scanty, inactive	Scanty, inactive	Scanty, inactive	Nil
Gonadotrophins as mg. IRP-HMG/24 hr.	5,30,5,20	10,20,5,5	10,20,5	5,20,5,5	20,5,5,5,10
Other conditions	Anorexia nervosa at 28	Empyema at 3	Nil	Nil	Nil

Table I.A Clinical data relating to patients before the first treatment

FIG.2A



Mean, maximum and minimum figures (shaded) for the excretion of oestriol by healthy women in (A) daily and (B) selected specimens of urine. The first day of menstruation is adjusted to occur on day 24.

In view of the amount of work involved in following each month of treatment in such a detailed way that Brown did to establish normal excretion values, it was decided to collect eight 24hr. samples of urine, these specimens were pooled in pairs and single specimens were collected at the beginning and end of each cycle. When the results for similar samples were taken from the figures of (Brown, 1959) and treated in the same way the mid cycle peak of oestriol was reduced. The resulting pattern is shown in Fig. 2B.

The normal pattern of excretion for pregnanediol is based on figures for daily excretion by 16 women aged 16 to 47 years with normal menstrual cycles reported by (Klopper, 1957). The figures for different age groups are shown in Figure 3A. The pattern of excretion by the women in the youngest age group, all of whom were single, was lower then the others. There was only one other single woman. She was in the next age group and her excretion was also low and the figures for these five women have been excluded. The mean, maximum and minimum figures for the remaining 11 are shown in Figure 3B. Figure 3C shows the effect of using the same pooled samples of urine for pregnanediol as was used in Figure 2B for oestriol.

The principal characteristics of this excretion pattern is the high plateau level in the luteal phase of the cycle. The increased values were maintained for about 10 days when they fall prior to menstruation. Comparison with Figure 2B shows that the primary peak in oestriol occurred when little change occurred in the pregnanediol level but as the oestriol level fell after the primary peak the pregnanediol level increased. The secondary oestriol peak, when it occurred, coincided approximately in time with the pregnanediol plateau. Both fell together prior to menstruation.

From the excretion patterns of both pregnanediol and oestriol during the normal cycles it can reasonably be assumed that this type of pattern could be a suitable model to try to induce using gonadotrophin stimulation in women that do not normally ovulate. The experiments described were designed with a view to induce this model pattern of steroid excretion.

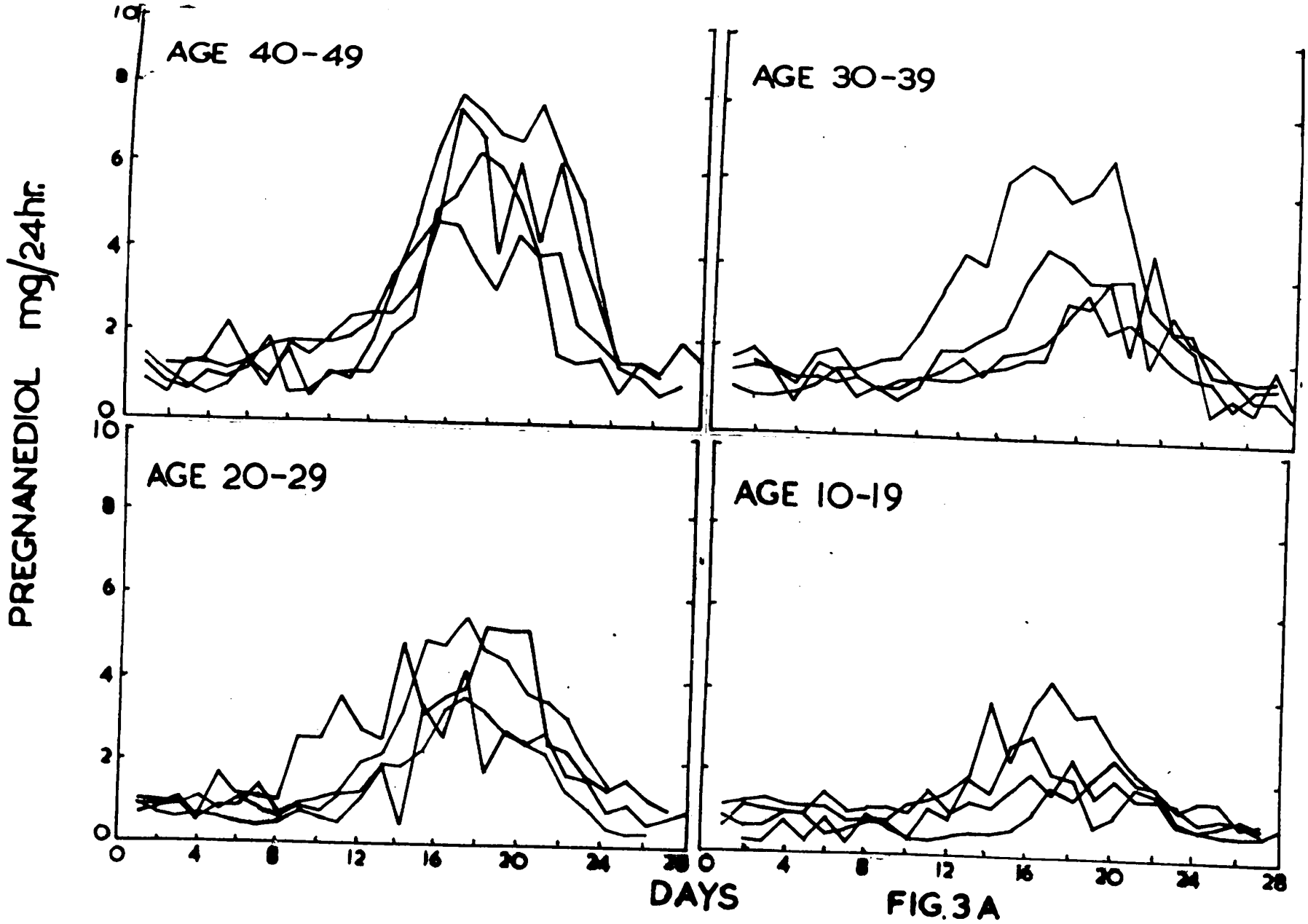


FIG. 3 A

The daily excretion of pregnanediol by healthy women of different ages.

The first day of menstruation is adjusted to occur on day 24.

The Design and Analysis of the Experiments:

The experimental trials were designed to study the effects of a number of different factors simultaneously in a single set of trials. Each set has been called an experiment.

In Experiment 1, the trials were arranged in the form of a Latin Square (Davies, 1960). Its objective was to compare the effects of different preparations of F.S.H. after eliminating possible bias due to differences between patient and between succeeding months of treatment. Experiment 1A was of simpler design, all the patients being given the same treatment for one month. It provided further information on the differences between patients.

The trials in Experiment 2 were also arranged in the form of a Latin Square. Here, the effects of changing both the total dosage and the daily dosage of F.S.H. were examined. As in Experiment 1, possible bias due to differences between patients was eliminated.

The factors examined in Experiment 2 did not appear to be acting independently of one another and it was considered likely that the factors to be studied in the next set of trials would also interact. These factors were the total dosage and the number of injections of H.C.G., the dosage of F.S.H., and the timing of the injections of H.C.G. relative to injections of F.S.H. Experiment 3 was therefore arranged to follow a half-replicate factorial design which enabled the effects of the above factors and some of their interactions to be assessed independently. Once more, possible bias due to different responses from patient to patient was eliminated.

The details of each experiment are described in association with their results since the design of succeeding experiments partly depended on these results. After the completion of the set of trials each patient was treated with the particular treatment that appeared to offer the best chances of producing an ovulatory type of steroid pattern.

The effects of the treatment on the amounts of oestriol and pregnanediol excreted have been assessed by analysis of variance. The object of the analysis was to isolate those differences in the experimental results which could not reasonably have occurred by chance from random fluctuations. Such differences are said to be statistically significant.

METHODS

AND

MATERIALS

METHODS AND MATERIALS.

Reagents and Materials.

Acetic acid A.R. (B.D.H.) was distilled from chromium trioxide and acetic anhydride.

Acetyl chloride and dimethyl sulphate A.R. (B.D.H.) were both distilled.

Furfuraldehyde A.R. (B.D.H.) was distilled twice. A solution was prepared 0.56% (V/V) in 50% (V/V) acetic acid which was stored at 4°.

Sodium bismuthate ($NaBiO_3$) laboratory reagent (B.D.H.) about 90%.

Sodium borohydride ($NaBH_4$). L.Light & Co. Colnbrook.

M-dinitro benzene (B.D.H.) "specially purified for 17-ketosteroids".

Pyruvic aldehyde (L. Light & Co., Ltd.) 25% solution in water.

Petroleum ether A.R. (B.D.H.) Boiling range (60-80°) and benzene (A.R.) were shaken with H_2SO_4 and allowed to stand overnight. They were washed free of acid and distilled through a fractionating column, the first and last fractions of each solvent were discarded.

Chloroform A.R. (B.D.H.) was distilled from potassium carbonate and stored in a dark bottle.

Diethyl ether A.R. (B.D.H.) was washed once with a saturated solution of ferrous sulphate followed by successive washings with water. It was distilled through a fractionation column and used soon after distillation. Ether recovered during the experiments was redistilled before use.

Other reagents were of analytical grade unless otherwise stated.

Silica Gel: (L. Light & Co., Ltd.) 100 - 200 mesh size, Davison, U.S.A. - Grade 923.

Alumina for androstenol. Type H (Peter Spence & Co., Ltd. Widnes) was partly deactivated by adding 3-4% water. Alumina used in the estimations of oestriol, pregnanediol and dehydroepiandrosterone (Savory & Moore Ltd) 100/150 mesh size, was adjusted by adding water until the desired activity was attained.

β-glucuronidase:

B-glucuronidase was prepared, to powder Stage B, by the method of Dodgson & Spencer (1953). Subsequently, however, ethanol was used instead of acetone to precipitate the enzyme as this had the advantage of giving a cleaner powder. The dry powder was stored in a refrigerator at 4° and the enzyme activity was determined at regular intervals using the method of Fishman, Springer & Brunetti (1948).
Sulphatase activity of the preparation was not estimated.

Chromatographic columns:

These columns were 1cm. x 10cm. and had a B14 cone joint at the resevoir end into which a B14/B24 adaptor was placed to act as resevoir. The absorbent material was supported by a disc of sintered glass of porosity 0. The column was filled with the solvent required by the particular method and the absorbent run in to the column. The column was then tapped gently and a small quantity of silver sand (acid washed) was added to prevent any disturbance of the absorbent when extracts were added. The solvent was run to the level of the sand before any extracts were added. Flow rates of solvents were regulated by the taps fitted to the columns and the rate was adjusted to that recommended for each individual method.
All glass apparatus was used throughout to avoid contamination from cork or rubber. Glassware was generally cleaned by immersing in chromic acid overnight and rinsing with dilute acid sulphite solution and finally water. Separating funnels were cleaned generally by rinsing in a solution of 50% HCL containing about 2% of $FeSO_4$, followed by several rinses of water.
Perspex agglutination trays were supplied by R. B. Turner & Co., Ltd.

Methods used for Steroid Estimations:

17-Oxogenic steroids.

A procedure based on the general method of Appleby, Gibson, Norymberski & Stubbs (1955) was followed.

4ml. of urine was added to a glass stoppered test tube containing 20mg. $NaBH_4$ and the solution was allowed to stand overnight. 4ml. of acetic acid was added and after 5 min. 1g. $NaBiO_3$. The tube was shaken for

30 min in the dark. Then 12ml. of a 12% sodium bisulphite solution (W/V) was added and the tube shaken on the mechanical shaker for a further 5min. 4ml. of H Cl. was added and the tube stood in a boiling water bath for 10min. On cooling the tube 15ml. of ethylene dichloride was added and the contents shaken for 5 min. The solvent layer was allowed to separate out and the acid layer was removed by suction. The solvent was washed successively with 50% H Cl. 15ml., water 15ml., 2N-N_aOH and water until all traces of alkali were removed. The solvent was evaporated after the addition of a little ethanol and a few pieces of anti-bump granules.

Colorimetry:

To the dry extract 0.4ml. of 1% metadinitrobenzene in ethanol was added followed by 0.2ml. of 2.5 N-KOH in methanol. The reagents were mixed by rotation of the tube and allowed to stand for 1 hr. in the dark. A pair of standard tubes, each containing 100µg. (dehydroepiandrosterone) and a blank containing reagents only were set up. After standing for 1 hr. 5ml. of ethanol was added to each tube and the extinction (E) read for each analytical solution against the blank at 520 and 430 mµ. A Unicam SP. 600 was used for all colorimetry. The E values of the analytical solutions were corrected by substitution in the formula as recommended by the Clinical Endocrinology Committee of the Medical Research Council (1951).

$$E\ cor. = \frac{E\ 520 - (0.6 \times E\ 430)}{0.73}$$

All results were expressed in terms of D.H.A, as mg. equivalents/24 hr.

17-Oxosteroids.

1.5ml. of HCL was added to 10ml. of urine in a glass stoppered test tube and heated in a boiling water bath for 10min. After cooling 15ml. of ethylene dichloride was added and the contents shaken for 5min. The acid layer was removed by suction and the ethylene dichloride was washed with 15ml. water, 15ml. 2N-N_aOH and water until the last wash was neutral to litmus. The remainder of the procedure is similar to that described for the 17-oxogenicsteroids. Results were expressed, in terms of a DHA standard, as mg. equivalents DHA/24hr.

Pregnanetriol:

The method of Morris (1959) was employed. 50mg. $NaBH_4$ was added to 10ml. of urine in a glass stoppered test tube and the contents allowed to stand overnight at room temperature. 10ml. of acetic acid, and 2g. $NaBiO_3$ were added and the tube shaken for 2 hr. in the dark. 8ml. of 12% $Na_2S_2O_5$ was added and the contents cooled. 4ml. of H Cl. was then added and the contents of the tube heated for 10min. in a boiling water bath. The solution was cooled and extracted in 30ml. of ethylene-dichloride by shaking for 3-5 min. on a mechanical shaker. The urine layer was removed by suction. The ethylene-dichloride was washed with 30ml. quantities of 50% H Cl, water, 2N-NaoH and water until neutral. A little ethanol and some anti-bump granules were added and the solvent was evaporated.

Chromatography:

The dry extract was dissolved in 10ml. of 25% ethyl acetate / petroleum ether (V/V) and applied to a chromatographic column containing 2g. silica gel set up in the same solvent. The test tube was rinsed with a further 2 x 5ml. of the same solvent, each addition was made to the column when the level of the previous extract had reached the level of silica gel. The first 20ml. of the effluent was discharged. Then 20ml. of 35% ethyl acetate / petroleum ether was added and the eluate was collected. This was called Fraction II and contained the oxidation product of pregnanetriol. Further elution of the column with 20ml. of 65% ethyl acetate / petroleum ether gave the corresponding 11-oxygenated 17-oxosteroids. This fraction was called Fraction III. The solvent was evaporated and both fractions were treated in a similar manner for colorimetry.

Colorimetry:

To each tube o.4ml. of metadinitrobenzene in ethanol and o.3ml. aqueous 8N-KOH were added. Two tubes containing 20µg. aetiocholanolone were included as standards. The reagents were mixed by rotation and allowed to stand for 25min. in the dark. 2ml. of 75% ethanol was added and the extinctions of the solutions were read against a blank containing reagents only at 440, 520 and 600 mµ. The Allen correction was applied to analytical tubes and to the standards.

Both fractions II and III are expressed in terms of mg. of aetiocholanolone/24hr. R, ratio of the two fractions was derived by dividing the value of fraction II by value of fraction III (Hill, 1960).

Pregnanediol.

The method of Klopper, Michie & Brown (1955) was used except that a smaller volume of urine was hydrolysed and the volumes of reagents used were reduced accordingly.

Hydrolyses and Extraction:

1/40th. of a 24 hr. specimen of urine was made up to 75ml. with water and added to a round bottomed flask containing 25ml. of toluene and anti-bump granules. The mixture was boiled under reflux and 7.5ml. H Cl. was added. The urine was boiled for a further 10 min. cooled and the toluene layer separated in a separating funnel. The urine layer was extracted in a further 25ml. of toluene and was then discarded. The combined toluene extracts were washed with 12.5ml. 25% NaCl in N-NaOH. The alkali layer was discarded with the curdy precipitate at the liquid interface. The toluene layer was transferred back to the original flask used for hydrolysis and shaken for 10 min. with a mixture of 5ml. of 5N-NaOH and 20ml. of 5% $KMnO_4$ (W/V). The mixture was transferred back to the separating funnel and the permanganate layer was discarded. The toluene layer was washed with water until it was free of the purple colour.

Chromatography:

The toluene extract was then applied to a chromatographic column containing 3g. of alumina in benzene. The column was first washed with 25ml. of 0.8% ethanol in benzene (V/V) and pregnanediol was then eluted in 12ml. 3% ethanol in benzene. The solvent was evaporated in a water bath. 2ml. of dry benzene was now added and warmed gently until all of the steroid residue had dissolved. 2ml. of acetyl chloride was then added to each tube and the contents left at room temperature for 1 hr. 25ml. of petroleum ether was added and the solution was transferred to a separating funnel, washed once with 50ml. water and once with 25ml. of 8% $NaHCO_3$, followed by 25ml. of water. When all traces of water had been removed the petroleum ether extract was added to a second chromatographic column containing the same

quantity of alumina but using petroleum ether instead of benzene. When the extract had reached the level of the alumina 15ml. of benzene was added and the pregnanediol diacetate was eluted. The solvent was evaporated and the tube was stood in a desiccator for 1 hr. at room temperature.

Colorimetry:

10mg. of sodium sulphite was added to each tube followed by 5ml. of H_2SO_4. Blank and standard tubes were treated in a similar manner. All tubes were stood overnight and the extintion of each read at 430 mμ. Results were calculated in terms of an authentic standard which had been brought through the method and was corrected for the difference in molecular weight between pregnanediol and its diacetate. Recoveries and differences between duplicates were similar to those of Klopper et al. (1955).

Oestriol.

Oestriol was measured by the method of Brown (1955).

Hydrolysis and Extraction:

200ml. of urine was boiled under reflux in a round bottomed flask 30ml. of H Cl. was added and boiling continued for 60 min. When cold the urine was extracted once with 200ml. of diethyl ether in a large separating funnel, both layers were allowed ample time to separate and the urine layer was run off and further extracted twice with 100ml. of diethyl ether.

Alkali washings:

The combined ether extracts were transferred to a separating funnel and washed with 80ml. of NaOH/$NaHCo_3$ buffer. (150ml. of 5N-NaOH added to 1 litre of 8% $NaHCO_3$ pH approx. 10.4). The bicarbonate layer was discarded. 20ml. of 2N-NaOH was added to the ether layer and the funnel was shaken vigorously. After standing for 5 min. 80ml. of 8% $NaHCO_3$ was added and the funnel again shaken thoroughly. After separation of the two layers the alkali was discarded and the ether was washed with a further 5ml. of 8% $NaHCO_3$ followed by 5ml. of water. When all the water had been drained off, the ether was transferred to a clean dry round bottomed flask and was evaporated on a water bath. The ether was recovered and was used again without purification for further extractions. The last traces of ether

were removed by tilting the flask on its side while still warm.

The dry extract in the flask was dissolved in 1ml. of ethanol and 25ml. benzene was added. The flask was rotated and the contents were transferred to a small separating funnel that contained 25ml. of petroleum ether. The flask was rinsed with 25ml. of water which was added to the separating funnel.

Methylation:

The contents of the separating funnel were shaken and the oestriol was extracted from the solvent layer into the water layer. This water layer was run into a large glass stoppered test tube (methylation tube) and the solvent layer was extracted further with 25ml. of water which was also added to the tube. 4ml. of 5N-NaOH and 0.9g. boric acid were added to the methylation tube and the contents were shaken until all the boric acid had dissolved. 1ml. of dimethyl sulphate was added (to the methylation tube) and the contents were shaken until all the dimethyl sulphate had dispersed. The tube was heated at 37° for 30 min. A second methylation was brought about by adding 2ml. of 5N-NaOH to the methylation mixture followed by 1ml. of dimethyl sulphate. After shaking, the tube was allowed to stand at 37° overnight in an incubator. The methylation mixture, after cooling, was added to a small separating funnel and the tube was rinsed out with 25ml. of benzene which was also added to the separating funnel. 2.5ml. of H_2O_2 and 10ml. of 5N-NaOH were also added and the separating funnel shaken thoroughly. The alkali was discarded and the benzene was washed twice with 5ml. water.

Chromatography:

When the last traces of water had been drained off the benzene extract was applied to a chromatographic column containing 2g. of alumina in benzene. After the benzene extract had percolated through the column 12ml. of 1.4% ethanol/benzene was added to the column and a band of pigment eluted. Oestriol methyl ether was eluted in 16ml. of 2.5% ethanol/benzene. 0.2ml. of 1% quinol in ethanol was added to the tubes containing the oestriol methyl ether and the solvent was evaporated off. An identical amount of quinol was also added to a blank and two tubes containing standards.

Colorimetry:

3ml. of Kober reagent (2% (W/V) ouinol in 76% H_2SO_4 V/V) was added to each tube, and after the contents were mixed by rotation the tubes were placed in a boiling water bath for 20 min. During the first 6 min. the tubes were shaken three times to ensure dissolution and mixing of the contents. After 20 min. the tubes were removed, cooled, 1ml. of water was added to each and heated in the boiling water bath for a further 10min. The tubes were cooled in ice water for 10 min. and the extinctions read against a reagent blank at 480, 516 and 552 mμ.

The Allen (1950) correction was applied to the extinctions of both the analytical and standard tubes.

E cor. (analytical) = 2 E (516) - (E 480 + E 516)

Results were calculated from the standards of oestriol methyl ether and were converted to oestriol by correcting for the differences in the molecular weights. The method was checked by adding oestriol standard to acid hydrolysed urine when-ever new batches of solvent or alumina were used.

The recovery of oestriol added to acid hydrolysed urine was similar to the recovery described by Brown (1955).

Estimation of Androstenol.

The method of Brooksbank & Haslewood (1961) was used.

Hydrolysis and Extraction:

1/10th, or in cases of low androstenol level 1/5th of a 24 hr. specimen of urine was adjusted to approx. pH 4.5 with a few drops of H Cl. 4M-sodium acetate buffer was added, 6.25ml. to every 100ml. of urine. Also limpet powder was added to give a concentration of B-glucuronidase of 1,600 Fishman units/ml. of incubation mixture and the urine stood at 37° for 70 hr. in an incubator. After incubation the urine was extracted with an equal volume of diethyl ether, followed by 2 extractions in the same solvent using 0.5 vols. The combined ether extracts were washed with 0.1 vol. of N-NaOH followed by water (3 x 0.1 vols). Any precipitated material from the incubation flask was washed thoroughly with diethyl ether prior to the alkali wash. After the diethyl ether extract had been drained of excess water it was dried by adding about 10g. of Na_2SO_4 and left for 1 hr. The ether was filtered into a long necked round bottomed flask and the filter paper

and Na_2SO_4 washed twice with 25ml. of ether. The ether was evaporated on a warm water bath and the last traces were removed by standing the flask in a vacuum desiccator.

Chromatography:

One solvent system was used throughout both to apply and elute the androstenol fraction. The dry extract was dissolved in 4ml. of a mixture of benzene/petroleum ether (V/V) and applied to a chromatographic column containing 5g. of alumina in the same solvent system. The extract was applied to the column slowly so that little spreading at the origin took place. This was necessary so that the androstenol eluted in a sharp band. The flask was rinsed with 2 x 2ml. of solvent and the rinsings also applied to the column. The column was allowed to run slowly (30ml./hr.) and the eluate containing androstenol was determined by plotting the elution pattern either of an extract of urine that contained large quantities of androstenol or by adding authentic standard to the column. It was found that androstenol was eluted after 65ml. of solvent had passed through the column, and the fraction eluted between 65 - 100ml. was collected in a 150ml. long necked flask. Constant checks were carried out on the elution of androstenol and the solvents were made up in large batches to avoid any source of variation. Dry solvents were used for chromatography.

The solvent was evaporated under reduced pressure on a water bath and the final traces were removed by standing in a vacuum desiccator.

Colorimetry:

The dry extract was dissolved in the flask by adding 3ml. of 0.5% resorcylaldehyde in acetic acid (W/V). The flask was stoppered and rotated so that all traces of the extract were dissolved and the solution obtained would be homogeneous. Then, two 1ml. samples were pipetted into colour reaction tubes (10 x 1cm.) and 1ml. of 5% H_2SO_4/acetic acid (V/V) was added with careful mixing to each tube. Androstenol standards and a blank were prepared using the same volumes of both reagents.

The tubes were placed without delay in a boiling water bath for 8 min. and then cooled in ice water in the dark. After 20 min. the solutions were diluted by adding 2ml. of acetic acid to each and E^{o} measured at

575 and 530 mμ against a reagent blank. The colour correction of Gibson & Evans (1937) as adapted by Brooksbank & Haslewood (1961) was used. The corrected reading E(cor) for the androstenol fractions was calculated by substitution in the formula as derived by Brooksbank & Haslewood (1961).

$$E\ 575\ (cor) = \frac{K_1\ E575\ (obs) - E\ 530\ obs.}{K_1 - Ka}$$

In this method of colour correction, the limiting value of E530/E575 was taken as 2, (K_1) that was where an extract contained representative amounts of interfering chromogen and little androstenol. The value for pure androstenol E530/E575 (K_0) derived in this work was 1.04, so that $K_1 - K_a = 2 - 1.04 = 0.96$.

Substitution in the above formula gave

$$E\ 575\ cor = \frac{2\ E575\ obs - E\ 530\ obs.}{0.96.}$$

Colormetric duplicates were estimated.

Results were expressed as the mean of duplicate estimations. Androstenyl B-glucosiduronic acid was not available for recovery experiments but experiments based on adding pure androstenol to urine before and after hydrolysis gave similar results to those attained by Brooksbank & Haslewood (1961).

Dehydroepiandrosterone.

Dehydroepiandrosterone was estimated by the method of Fotherby (1959).

Hydrolysis and Extraction:

40ml. of urine was heated for 6 hr. under reflux on a boiling water bath. The urine was used when fresh and the pH was not adjusted. After cooling, the urine was extracted with 40ml. of benzene and the benzene layer was washed three times each with 20ml. of water. 35ml. of the benzene extract was evaporated to dryness under reduced pressure on a warm water bath. 8ml. of benzene was added to the flask, the contents were mixed well and the benzene transferred to a chromatographic column containing 3g. of alumina in benzene. The flask was rinsed with a further 2 x 4ml. of benzene and these rinsings were also added to the column. The column was washed with a further 10ml. of benzene, this step ensures the removal of some pigment band and dehydroepiandosterone was eluted in 30ml. of 0.1%

ethanol in benzene (V/V). The eluate was evaporated to dryness and the residue together with standard dehydroepiandrosterone were subjected to the modified Pettenkoffer reaction (Munson, Jones, McCall & Gallagher, 1948).

Colorimetry:
The extract was dissolved in 0.2ml. acetic acid and 0.8ml. of 0.56% furfuraldehyde in 50% acetic acid was added followed by 3ml. of 8M-H_2SO_4. The contents were mixed and the tubes were incubated at 68° for 12 min. After colour development and cooling E° of each tube was read against a blank containing reagents only at 620, 660 and 700 mμ. The correction formula of Allen (1950) was applied to both standard and analytical tubes.

Recovery:
Recovery experiments were performed by adding DHA - sulphate to urine, the results of these experiments were calculated after subtraction of the endogenous DHA already present. The results obtained were similar to those obtained by (Fotherby, 1959).

Preparation of Gonadotrophins from human pituitary glands.

Extracts of human pituitary glands were supplied as acetone dried powder by Dr. A. Korner (University of Cambridge, Dept. of Biochemistry).

The acetone dried powder was extracted in 10% ammonium acetate-ethanol (60 : 40: V/V) and then precipitated by the addition of ethanol until the concentration was 80% (V/V). The fractionation scheme is shown on the flow sheet (Fig. 4). Amounts of 100-200mg. were applied to columns (20cm. x 1cm.) of carboxymethyl cellulose in 0.01 M-ammonium acetate pH 6.1. Fraction CM1 was not absorbed under these conditions and fraction CM2 which was absorbed was eluted in 1M-ammonium acetate pH 6.1 CM1 was further fractionated on a column of diethylaminoethyl cellulose (10cm. x 1cm) by the method described for urine (Butt, Crooke & Cunningham, 1959). The fraction eluted in 0.2M-ammonium acetate was then put directly through a column of calcium phosphate (10cm. x 1cm.) and the fractions eluted in 1mM-and 0.02M-disodium phosphate were collected, dialysed and reprecipitated by the addition of 5 vol. of ethanol.

CM2 was refractionated on carboxymethyl cellulose (20cm. x 1cm.) by stepwise elution in ammonium acetate (0.01 - 0.0M). Gonadotrophin was detected only in the fraction eluted in 0.1 M-ammonium acetate. In all chromatographic work the elution of protein was detected by observing the U.V. absorption of the eluate at 280mμ in a Unicam spectrophotometer S.P. 500.

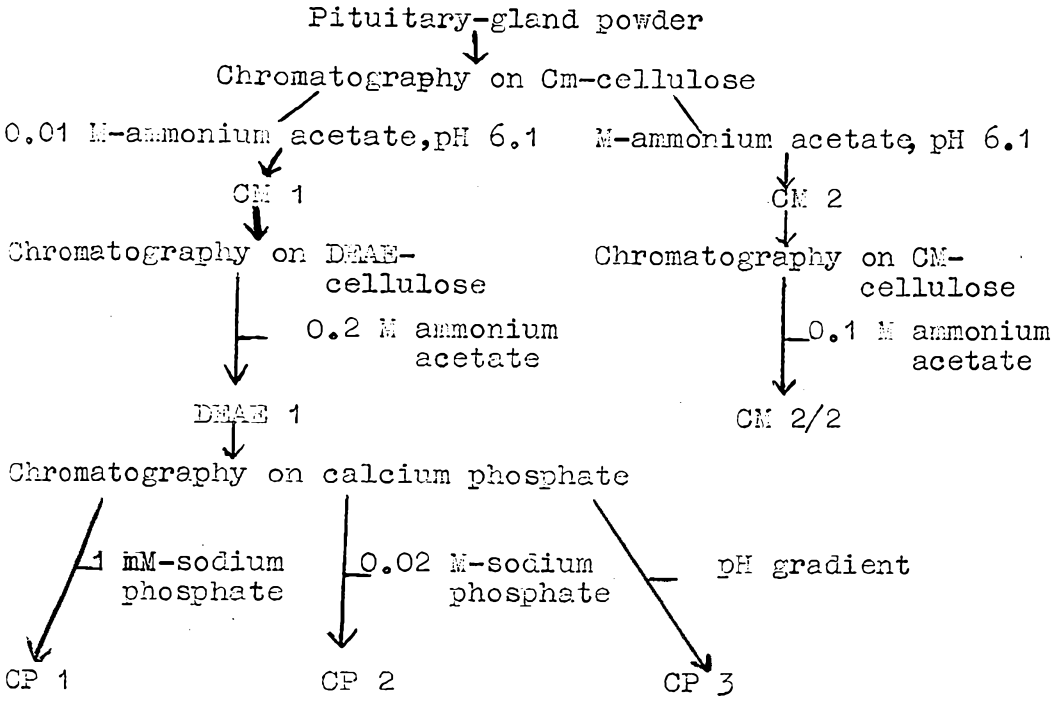


Fig.1.

Scheme for the chromatographic separation of pituitary-gland powders with carboxymethyl (CM)-cellulose and diethylaminoethyl (DEAE)-cellulose and calcium phosphate (CP).

Other preparations used.

Gonadotrophins from urine were obtained from commercial sources, they were Organon 613 prepared from post menopausal urine and human chorionic gonadotrophin (HCG) obtained from Paines & Byrne Ltd.

Human growth hormone:

(HGH) batch No. 83, prepared from acetone dried human pituitary powder by the Raben method.

Thyrotrophic hormone (TSH) and adrenocorticotrophic hormone (ACTH) were commercial preparations

Biological Methods.

Ovarian augmentation assay.

In this assay an excess of luteinizing hormone in the form of human chorionic gonadotrophin is added to

Table II.

Preparation	Ovarian Augmentation assay	Ovarian Ascorbic Acid depletion assay
CM 1	183(84 - 355)	265(108 - 580)
CP 2	1,014(753 - 1,555)	420(190 - 970)
CP 1	4,300(3,200 - 5,700)	906(588 - 1,397)
613	81(30 - 106)	77(25 - 192)

Relative potencies of gonadotrophins

the test material (Brown, 1955) and the combined weight of the ovaries is the criterion of the response. The procedure is sometimes termed the 'augmentation assay' and is thought to be fairly specific for FSH (Brown, 1959).

Immature female mice of an albino strain supplied by a dealer accredited by the Medical Research Council (Mr. W. Clarke of Oldham) were used. The gonadotrophic material to be assayed and the added HCG were injected subcutaneously in five equal doses, each in 0.5ml. physiological saline, over a three day period. Injections were made twice a day on the first two days and once on the third. The animals were killed approx. 72 hr. after the first injection and the ovaries were dissected and weighed.

Ovarian ascorbic acid depletion assay.

Parlow (1958) described a method for the estimation of LH activity in pituitary extracts in which intact rather than hypophysectomised animals were used. The end point of the assay depended on the depletion of ascorbic acid in the ovaries of pseudopregnant rats. This assay was claimed by Parlow to be highly specific and sensitive for LH. The technique used was essentially that of Parlow (1958, 1961) with a few minor modifications. The procedure was carried out in two stages -

Pretreatment of animals:

Immature female rats weighing 40 - 50g. were used. Each animal received a subcutaneous injection of 50 i.u. of pregnant mare serum gonadotrophin (PMS Gestyl, Organon) and 56 - 65 hr. later a subcutaneous injection of 25 i.u. HCG. (Pregnyl-Organon). Both preparations were dissolved in physiological saline and each injection was given in a total vol. of 0.5ml.

As a result of this treatment the animals became pseudopregnant and showed heavily luteinized ovaries ranging in weight from approx. 80 - 100mg.

Bioassay of preparation:

This was performed on the 5th or 6th day after the injections of HCG. The rats were injected intra-peritoneally with the gonadotrophic material to be assayed dissolved in 0.2ml. of physiological saline. The animals were killed 4 hr. later by cervical dislocation. The right ovary from each animal was removed, rapidly cleaned on filter paper moistened with

2.5% metaphosphoric acid, and weighed. Ascorbic acid was estimated according to the specification of Dekanski & Harvie (1960). Each ovary was homogenized using a mortar and pestle with 2.5% meta phosphoric acid (W/V, freshly prepared) and a trace of sand. After a thorough grinding the homogenate was suspended in a total volume of 10ml. of 2.5% metaphosphoric acid and allowed to stand for at least 30min. At this stage the suspension could be left overnight in a refrigerator at 4° provided freshly prepared ascorbic acid standards were kept similarly. The suspension was centrifuged at 2,500 r.p.m. for 5 min. and 8ml. of the supernatant was taken for the estimation of ascorbic acid. 7.0ml. of 4.53% sodium acetate adjusted to pH 7.0 with acetic acid was added followed by 1ml. of 0.03% 2 : 6 dichlorophenolindophenol. This produced a pink colour the extintion of which was read at 520 mμ. A calibration curve for ascorbic acid in concentrations up to 200 ug./10ml. in metaphosphoric acid was prepared. The unknown values for the test solutions were interpolated and the results were expressed as μg. ascorbic acid/100mg. tissue.

Immunological Methods.

Immunological estimation of HCG in urine.

Production of antisera:

1g. of bentonite (B.D.H. Ltd.) was suspended in 100ml. of 0.9% sodium chloride solution. Coarse particles were allowed to settle out and were separated by decantation and discarded. 2.7mg. of a preparation of chorionic gonadotrophin (Leo Pharmaceutical Products, Copenhagen) equivalent to 4,400 units of International Standard was mixed with an equal weight of suspended bentonite. The volume was adjusted with the saline to 12ml. and 2ml. was administered intravenously to rabbits (Sandylops) twice a week for three weeks or more until a satisfactory antibody titre was obtained. The rabbits were bled seven days after the last injection and a few drops of 10% solution of sodium azide was added to the serum as a preservative and stored at 4°.

Red cell haemagglutination technique.

The reaction between anti HCG and red blood cells coated with HCG was used for the quantitative estimation of HCG in the urine of pregnant women.

Sheep cells preserved in Alsever solution were

obtained from Wellcome Research Laboratories Ltd. The cells were washed three times with normal sodium chloride solution and then treated with pyruvic aldehyde (25% solution, L. Light & Co., Ltd.). 1.6 vol. of this reagent was added to 3.0 vol. of sodium chloride solution and the pH was brought to approx. 7.0 with 10% sodium carbonate solution. 0.7 vol. of 0.15M-phosphate buffer pH8.0 and 1 vol. of 50% suspension of red cells were than added to the mixture and stored at 4° for two days with occasional mixing. The cells were thoroughly washed with sodium chloride solution and stored as a 10% suspension containing 0.1% of sodium azide as preservative.

Antigen was then attached to the cells. A portion (2ml.) of the 10% suspension of cells was centrifuged and the supernatant discarded. The cells were washed once with dilute buffer (sodium chloride-water-phosphate, 0.15M, pH 6; 10 : 10 : 1 x vol.) and then suspended in 1.5ml. of the same buffer containing 100 - 500 ug. of antigen. After heating at 50° for 1 hr. the cells were centrifuged and washed three times with sodium chloride solution containing rabbit serum (0.5%) and finally were suspended in 10ml. of sodium chloride solution containing 1% (V/V) of rabbit serum and 0.1% azide. Antiserum was heated at 56° for 30 min. to destroy complement and was then absorbed with sheep red cells as described by Read & Bryan (1960).

0.2ml. of sodium chloride was added to each well in a perspex agglutination tray and a series of doubling dilutions of urine and of a laboratory standard of HCG, starting at a concentration of 10 i.u./ml., were set up. A portion 0.2ml. of serum-sodium chloride solution was added to each well followed by 2 drops (0.05ml. approx.) of red cell suspension. After mixing, the cells were allowed to stand overnight. If no haemagglutination occurred the cells sank to the bottom of the well forming discrete buttons; if haemagglutination occurred the cells spread out evenly over the bottom of the well. Results were calculated by comparion between the haemagglutination in the standard and test rows and expressed as i.u. HCG/24 hr.

Statistical Methods and Presentation of Results.

Standard statistical procedures were employed both for analysis and design of experiments. Analysis of

variance was carried out as described by Davies (1960). Where ever possible results of experiments were shown graphically as well as graphic illustrations of the statistical analyses. The results of bioassays were calculated by the method of Gaddum (1953) and Borth (1960).

RESULTS

SECTION I

A shortened procedure for the estimation of Oestriol in Urine.

This section describes a method for the estimation of oestriol in urine based on the method of Brown (1955) and the modification of that method by Brown, Bulbrook and Greenwood (1957) and Salokangas and Bulbrook (1961).

Experiments and Results.

Hydrolysis:

In the method of Brown (1955) HCl.G. was used to hydrolyse the three oestrogens, oestriol, oestrone and oestradiol- 17β from their urinary conjugates. The hydrolysis involved adding 15ml. HCl.G. to 100ml. of urine and refluxing for 1 hr. Using the same method Brown and Blair (1958) showed that oestriol was liberated from its conjugate in urine in a much shorter time if the amount of acid used was increased. They found that adding 20ml. HCl/100ml. of urine liberated maximum quantities of oestriol in 30 mins. but the hydrolysis of the other two oestrogens required a longer time. Preedy and Aitken (1961) also found that 17.5% of HCl.G. with 45 mins. boiling resulted in maximum recovery of oestriol. In the method described here 20ml. of HCl.G. was added to 100ml. of urine which was boiled under reflux for 30mins.

Extraction:

It was found that a single extraction with an equal volume of diethyl ether was adequate to extract oestriol from acid hydrolysed urine but occasionally emulsions were formed between the 2 layers. These emulsions are not troublesome in the Brown method as they are dispersed by the large volumes of ether used. To overcome this difficulty, in the shortened procedure, sodium chloride was added to each separating funnel before the urine was extracted. This had the extra advantage that the increase in salt content of the urine altered the partition coefficient of oestriol in favour of the ether layer. The addition of sodium chloride was therefore incorporated in all estimations. The extraction and washings with buffer and alkali are identical to those used by Brown (1955) but the volumes were reduced by approximately one third. In the method of Brown the ether extract after washing with alkali and water was evaporated to dryness and the residue was taken up in benzene/petroleum ether (25/25 V/V).

Oestriol was extracted from the mixture with water, and oestrone and oestradiol with dilute alkali. Since the oestrone and oestradiol fraction was not needed in this short method it was decided to extract the oestriol directly from the diethyl ether layer by dilute alkali, so omitting the benzene/petroleum ether partition stage. In a later modification of the method of Brown (Brown, Bulbrook and Greenwood, 1957 b) used this step to extract oestriol from diethyl ether.

The extract of oestriol prepared by this procedure contained more pigment than is normally found in the corresponding oestriol fraction. This was partly due to the slight solubility of diethyl ether in alkali so that some ether that contained pigment was brought through by the alkali. This ether evaporated off during the methylation procedure and did not cause any trouble at the chromatography stage.

Colorimetry:

The conditions for the development of the Kober colour were identical to those described by Brown (1955). A greater sensitivity could be attained for the method if the volumes of reagents used were reduced. The work of Salokangas and Bulbrook (1961) showed however that the development of the Kober colour was reduced as the amount of urine residue increased and no improvement in the results were obtained even after extraction of the Kober colour into organic solvent.

Comparison of the established method with the shortened procedure using the original Kober colour without solvent extraction, showed clearly that at low concentrations of oestriol the shortened procedure over-estimated the quantities of oestriol present. This was due to the fact that the colours produced in the shortened procedure were observed to contain more pigment than the corresponding extracts obtained by the longer method. A further purification stage was therefore required in the method to remove this non-specific interference by pigment and to achieve this the final Kober colour was extracted into an organic solvent.

Bradshaw (1961) found that the addition of trichloroacetic acid (T.C.A.) to the final Kober solution of oestrone and oestradiol enabled the colour to be extracted into chloroform. The extracted colours

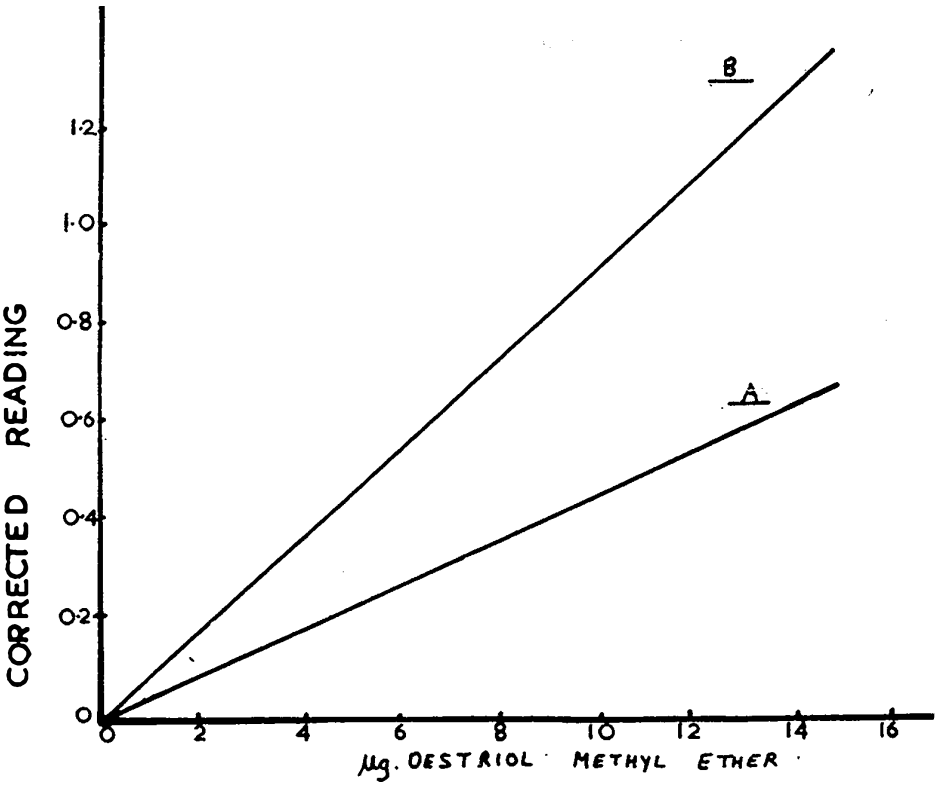
were found to absorb at longer wavelengths and had increased in intensity and were more stable than those extracted by the method of Ittrich (1958). Chloroform had the advantage of extracting less non-specific colour than tetrachloroethane (Aldercreutz, 1963). The extraction procedure of Bradshaw was therefore used to estimate oestriol.

This procedure was more suited for extraction than some of the other solvents used (tetrachloroethane, tetrabromoethane) which had unpleasant toxic fumes. This procedure of extraction into chloroform was found suitable for a method to be used in a routine laboratory where special fume cubboards are generally not installed. When this extraction was applied to oestriol methyl ether after development (of the Kober colour) it was found that the extracted colour exhibited an absorption maximum at 532mμ and had increased in intensity. Taking the absorption maximum at 532mμ and applying the Allen correction using extinction readings equidistant from the maximum on each side of the absorption spectrum, a straight line graph was obtained by plotting corrected reading distance (mμ) from the maximum. This satisfied the condition (O'Sullivan, 1958) for the validity of the Allen correction; so in the method, readings were taken at 502, 532 and 562 mμ and the Allen correction applied.

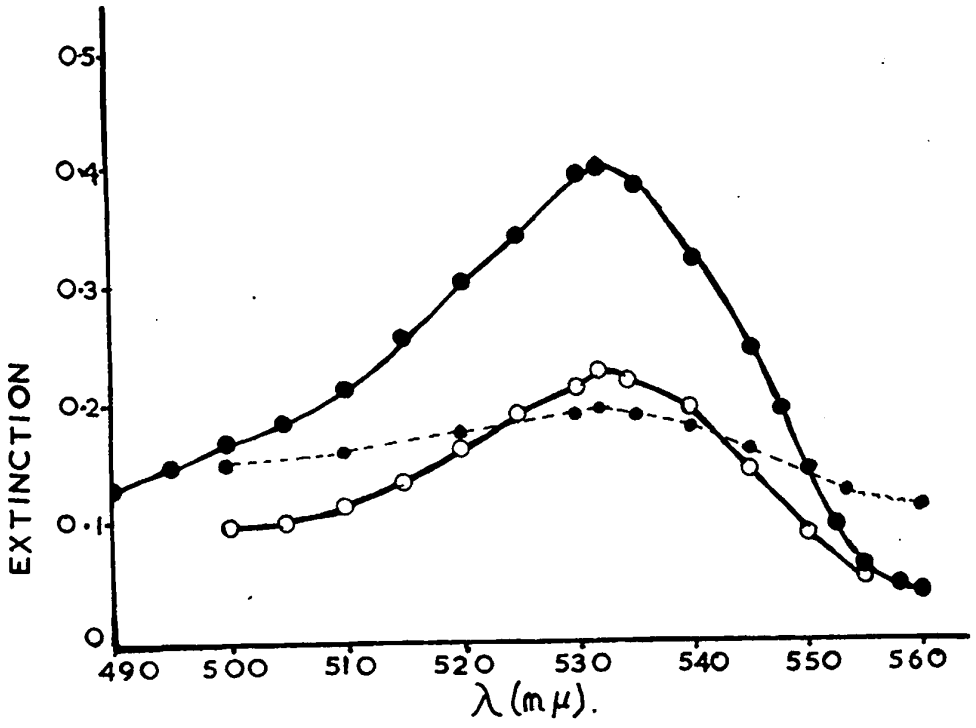
This change in wavelengths of the extracted colour of oestriol methyl ether led to a greater degree of specificity to the colour reaction and a greater degree of purity of the final colour. Considerable quantities of pigment were found coagulated at the interface of the acid and chloroform layers, this pigment was removed with the acid layer before colorimetric estimations. The absorption spectra and calibration curves for the shortened procedure (B) and the method of Brown (A) are shown.

Stability of the colour:

Salokangas and Bulbrook (1961) claimed that considerable fading took place when the Kober colour was extracted into chloroform following dilution of the original Kober colour with water. Similar results were found by Aldercreutz (1963) who reduced the degree of fading considerably by working with ice cold reagents. In this work it was found that after dilution of the Kober colour with trichloracetic acid the amount of fade



Calibration curves for oestriol methyl ether
(A) prepared according to the method of Brown (1955)
(B) prepared by the conditions described in the short method.



Absorption spectra of
(1) authentic oestriol methyl ether (closed circles)
(2) extract from pregnancy urine (open circles)
(3) extract from urine that contained only a small quantity of oestriol (broken line)

at 532mμ was negligible during the first 30min. when ice cold reagents were used for dilution and extraction. This small amount of fading in colour did not constitute any disadvantage to the method as never more than ten estimations were made at any one time and standards were incorporated with each batch. When the colour was extracted the tubes were protected from sunlight as much as possible and each batch estimated by a standardised procedure for centrifugation and colorimetric estimation.

Method in Detail

As in the method of Brown (1955) no preservative was added to the urine.

Hydrolysis and extraction:

100ml. of urine was boiled under reflux and when boiling 20ml. HCl was added and some alundum chips and boiling continued for 30min. The urine was cooled under running water and transferred to a separating funnel that contained 120ml. of diethyl ether and 16g. of sodium chloride. The funnel was shaken thoroughly and the two layers were allowed to separate. The acid layer was discarded and the ether was washed with 25ml. of $NaOH/NaHCO_3$ buffer (pH 10.4) which was discarded. 6.5ml. of 2N-NaOH was then added to the separating funnel and the contents were shaken. After allowing this alkaline layer to stand for 5 min. 25ml. of 8% $NaHCO_3$ was added and the flask again shaken thoroughly. The alkaline layer was discarded and the ether was washed with 5ml. of 8% $NaHCO_3$ to remove the last traces of the previous alkaline wash from the stem and tap of the separating funnel.

Methylation:

When the last traces of bicarbonate had been drained off, the ether was extracted twice with 25ml. of 1.6% NaOH which was added to a methylation tube (large ground glass stoppered test tube approx. vol. 100ml.). 0.9g. of boric acid was also added and the tube was stood at 37° in a water bath. When the boric acid was dissolved 1ml. of dimethyl sulphate was added to the tube which was then shaken so as to disperse the dimethyl sulphate. A few alundum chips were added to the methylation tube, they assisted in the evaporation

of the ether that had been brought through by the alkali. After standing the tube for 30min. at 37°, 2ml. of 5N.-NaOH was added followed by 1ml. of dimethyl sulphate. The mixture was again shaken and stood at 37° for a further 30min.

Extraction and chromatography:

The methylation mixture was cooled and added to a separating funnel that contained 10ml. of 5N-NaOH. 2.5ml. H_2O_2 was then added and the methylation tube was rinsed with 25ml. of benzene which was also transferred to the separating funnel. The funnel was shaken thoroughly and the alkaline layer separated. The benzene layer was washed with a further 2 x 5 ml. of water. When the benzene was finally drained free of water it was transferred to a chromatographic column that contained 2g. of alumina set up in benzene. When the level of the benzene had reached the level of the alumina the extract was added to the column. The first fraction was eluted in 12ml. of 1.4% ethanol/ benzene which was discarded and the second fraction, 16ml. of 2.5% ethanol/benzene was collected. 0.2ml. of 1% quinol in ethanol and some anti-bump granules was added to this fraction and the solvent evaporated.

Colorimetry:

The Kober reaction was performed on the dry extract by adding 3ml. of Kober reagent and heating for 20min. in a boiling water bath. The tubes were shaken twice during the first 6 min. of heating to dissolve the extracts. On cooling 1ml. of water was added and the tube heated for a further 10min. The tube was again cooled.

3ml. of this Kober solution was added to a small glass stoppered tube (Quickfit and Quartz) which contained 1.8ml. of ice cold trichloracetic acid. The two acid layers were mixed by gentle rotation and the tube was cooled again in ice. After 5min. 2ml. of ice cold chloroform was added and the tube was shaken for 30sec. Immediately it was centrifuged at approx. 1700g. for 2 min. to separate the two layers. The acid layer was removed by suction together with any coagulated pigment at the interface of the two liquids. The extinction was read at 502, 532 and 562 mμ. on a Unicam S?P.600/ against a reagent blank brought through the method. Standards were incorporated in each batch of

estimations and from the Allen correction the quantity of oestriol methyl ether and hence the amount of oestriol present was calculated.

Comparison of the modified method with the method of Brown (1955)

Urine from women with secondary amenorrhoea who were excreting small quantities of oestriol was pooled. Estimations were performed by the method of Brown and by the short procedure on the pooled sample of urine to which had been added different quantities of oestriol after hydrolysis. Each estimation was carried out in duplicate and the results by the short method are shown before and after extraction into chloroform (Table A).

Oestriol added/µg/24hr.	Brown 1955 A	Short Procedure Extracted. B.	Short Procedure Unextracted. C.
0	3.2	4.1	8.8
	6.8	1.7	4.8
4.5	10.0	6.7	10.7
	9.4	7.7	12.9
9.0	11.7	10.1	13.9
	11.2	10.0	14.5
13.5	13.1	13.3	17.7
	13.5	16.6	24.5
18.0	19.1	19.7	22.4
	19.4	19.2	22.6
22.5	22.2	23.9	30.0
	23.3	19.8	22.4
Mean % Recovery	81	87	98

TABLE A. The results of duplicate estimations and mean percentage recovery at different levels of oestriol using:-

A) The method of Brown 1955.

B) The short procedure.

C) The short procedure without colour extraction.

These results show that the estimates obtained by the short method agree reasonably well with those obtained by the method of Brown when the colour was extracted. Since the extraction procedure quantitatively extracts the Kober colour (Bradshaw, 1961) the reason

for the differences must be due to pigment present that is not accounted for by the Allen correction. After this initial experiment the extraction stage was therefore incorporated in all estimations.

Recovery experiments were performed by adding known quantities of oestriol to samples of pooled urine from women who excreted small quantities of oestriol. At low levels of oestrogen excretion the percentage error of a single estimation increased (Brown, Bulbrook and Greenwood, 1957) and therefore the range of recovery experiments was split into three groups. The results are shown in Table B.

Range. µg./24hr.	No. of duplicate estimations.	Mean % Recovery.	± S.D.
4.5 - 9.9	10	76	± 16.7
10.0 - 15.1	10	87	± 8.6
15.2 - 40.0	14	85	± 6.3

TABLE B. Results are shown as the mean percentage recovery ± standard deviation and are corrected for the endogenous blank value. The range of oestriol expressed as µg./24 hr. and the number of duplicate estimations performed are also shown.

Since oestriol conjugates were not available for recovery experiments and the object of the investigation was to derive a method to replace the method of Brown (1955) it was considered suitable to compare the results of duplicate analyses on aliquots of the same urine samples by both methods over a wide range of concentrations and to estimate the S.D. of each method in the range chosen.

An analysis of variance was performed on 20 duplicate estimations of the oestriol levels in urine from a group of different patients who excreted oestriol in the range 4.0 - 45.0µg/24hr. Results are shown in Table C. It was found that in this series there was no significant difference between the values obtained by the method of Brown and the shortened procedure. The standard deviation was derived for the range 4.5-130µg/24hr. for the shortened procedure and 4.5- 45µg/24hr. for the method of Brown.

Range µg./24hr.	(A) S.D. of Shortened Procedure	Number of duplicate estimations	(B) S.D. of Brown Method.	Number of duplicate estimations.
4.5 - 9.9	0.65	19	1.07	8
9.9 - 15	1.45	13	-	-
15 - 45	1.19	22	1.00	11
40 - 130	1.63	16	-	-

TABLE C. This table shows the standard deviation derived for the short method (A) and the method of Brown (1955) (B) over different ranges of oestriol values. The number of duplicate estimations performed are also shown.

The method of Brown was not investigated at levels above 45.0µg./24hr. as above this level the urine was diluted with water which gave better results by reducing the amount of pigment present at the colour development stage and the decrease is oestrogen destruction during hydrolysis (Brown & Blair, 1958). The results show that the shortened procedure appears to be suitable for estimating oestriol levels up to 130µg./24hr. without introducing any greater error.

Comparison of the two methods on urine from pregnant women.

The method of Brown (1955) was compared with the short method by carrying out duplicate estimations on 20 samples of urine from women at different stages of pregnancy. There was no significant difference between the results of the two methods.

The standard deviation of the methods was derived over the range of oestriol excretion encountered in early pregnancy.

Range mg./24hr.	% S.D.(B) Brown Method.	Number of duplicate estimations.	% S.D. of(A) shortened procedure.	Number of duplicate estimations.
0.98-12.0	0.4·0	20	0.36	30

TABLE D. This table shows the % standard deviation of the short method (A) and that of method of Brown (B) when used for estimating oestriol in urine from pregnant women. The number of duplicate estimations performed are also shown.

The results show that the short procedure is applicable to the range of oestriol levels encountered during pregnancy and the standard deviation of both methods is approximately the same.

RESULTS

SECTION II

RESULTS.

Presentation of results.

The results have been presented as follows: first, the individual responses of the patients have been recorded graphically in full in Figures 4, 8-10 and 14; second, the analyses of variance of the responses have been given in Tables in the text and the results described in the text and shown graphically with the 95% confidence limits in Figures 5-7, 11-13 and 15-19. Finally they have been summarised briefly in the Conclusions following each Experiment.

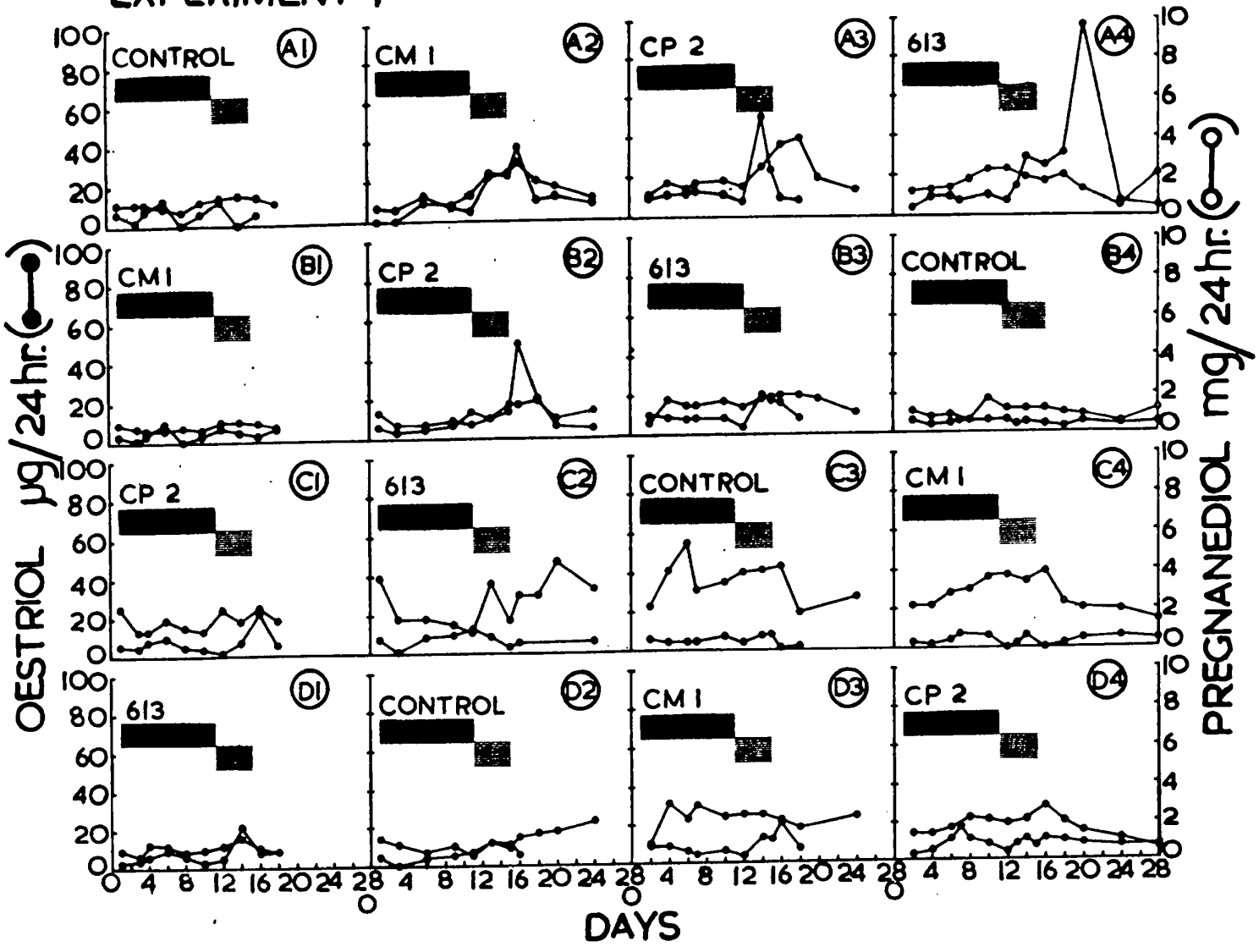
Experiment 1.
Comparison of the Effects of Different Gonadotrophin Preparations on Steroid Excretion.

In this experiment four patients, A,B,C and D, were given four treatments:- control (gelatin), CM 1, CP 2 and 613 over four courses of 28 days each. Each course of treatment was given in equally divided doses of F.S.H daily for ten days, followed by H.C.G. for four days. The daily doses of CM 1, CP 2 and 613 were all equivalent to 300mg. I.R.P.-H.M.G., measured by the augmentation method for F.S.H. Since they contained different amounts of luteinizing hormone, however, the amounts of total gonadotrophin varied between preparations. From the 11th to 14th day inclusive of each month each patient received 6,000 i.u. H.C.G. daily. Twenty-four hour samples of urine were collected on alternate days and oestriol and pregnanediol determined. The results are shown graphically in Figure 4. None of the patients responded in the control month despite the fact that they received 24,000 i.u. H.C.G.

Patient A responded to CM 1 with a slight mid-cycle rise in oestriol followed by a higher secondary rise and a slight rise in pregnanediol (Figure 4, A2). She responded to CP 2 with a normal mid-cycle rise in excretion of oestriol with a normal luteal rise of pregnanediol followed by menstruation (Figure 4, A3). With 613 she showed a secondary rise in oestriol which was even more pronounced than with CM 1 but there was no rise in pregnanediol (Figure 4, A4).

Patient B failed to respond to CM 1 (Figure 4, B1). She responded to CP 2 with a slightly delayed but good

FIG. 4



Excretion of oestriol and pregnanediol by patients A, B, C and D given different preparations of FSH or gelatin control and HCG during months 1 to 4.

FSH or control

HCG

In this and subsequent experiments the daily dosage of each substance is proportional to the area of shaded surface.

rise in excretion of oestriol and a poor rise in pregnanediol in the luteal phase (Figure 4, B2) and she showed a slight mid-cycle rise in oestriol with 613 but no change in pregnanediol (Figure 4, B3). She showed no change in the control month. She menstruated only after 613.

Patient C was found to have a high excretion of pregnanediol in the control period (Figure 4, C3). She showed no response to CM 1 (Figure 4, C4) but with CP 2 the pregnanediol fell to the normal level and she showed a slight rise in the excretion of oestriol (Figure 4, C1). With 613 the pregnanediol again fell to the normal range and rose in the luteal phase despite the absence of any rise in excretion of oestriol (figure 4, C2). She failed to menstruate with any treatment.

Patient D showed a slight rise in excretion of oestriol following CM 1 (Figure 4, D3) and 613 (Figure 4, D1), but there was no response to CP 2 (Figure 4, D4). There was no change in pregnanediol and she failed to menstruate with any treatment.

Analysis of Results.

Each cycle of 28 days was divided into four-day periods during the majority of which there was two measurements of oestriol and two of pregnanediol. The effects of the treatment were analysed separately for each of the first five four-day periods (i.e. the first 20 days of the cycle). The oestriol and pregnanediol results were analysed independently (Tables I and II).

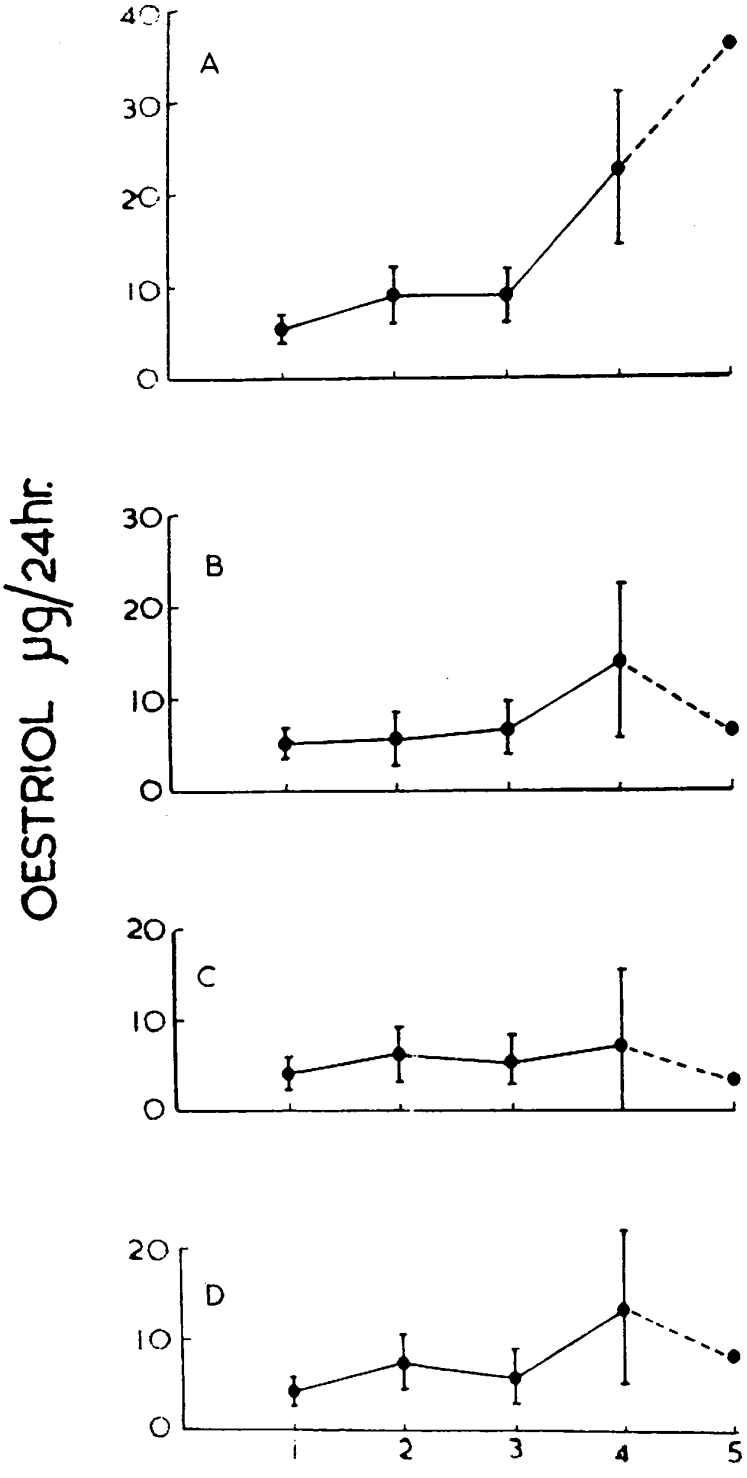
Oestriol:

Only the first four four-day periods were analysed. The fifth was omitted from the analysis because there were not sufficient observations, but the average for that period is shown with the others in Figures 5 and 6A.

Periods 1 to 3: The differences between the average figures for different patients and between the effects of different preparations were small and could be attributed to chance.

Period 4: The excretion of patient A was probably higher than that of patients B and D whose excretion was probably higher than that of patient C (figure 5). The average excretion following treatment with CM 1, CP 2 and 613 was higher than that following the control. The highest average excretion was after CP 2, although it was not significantly higher than that after CM 1 or 613 (Figure 6A).

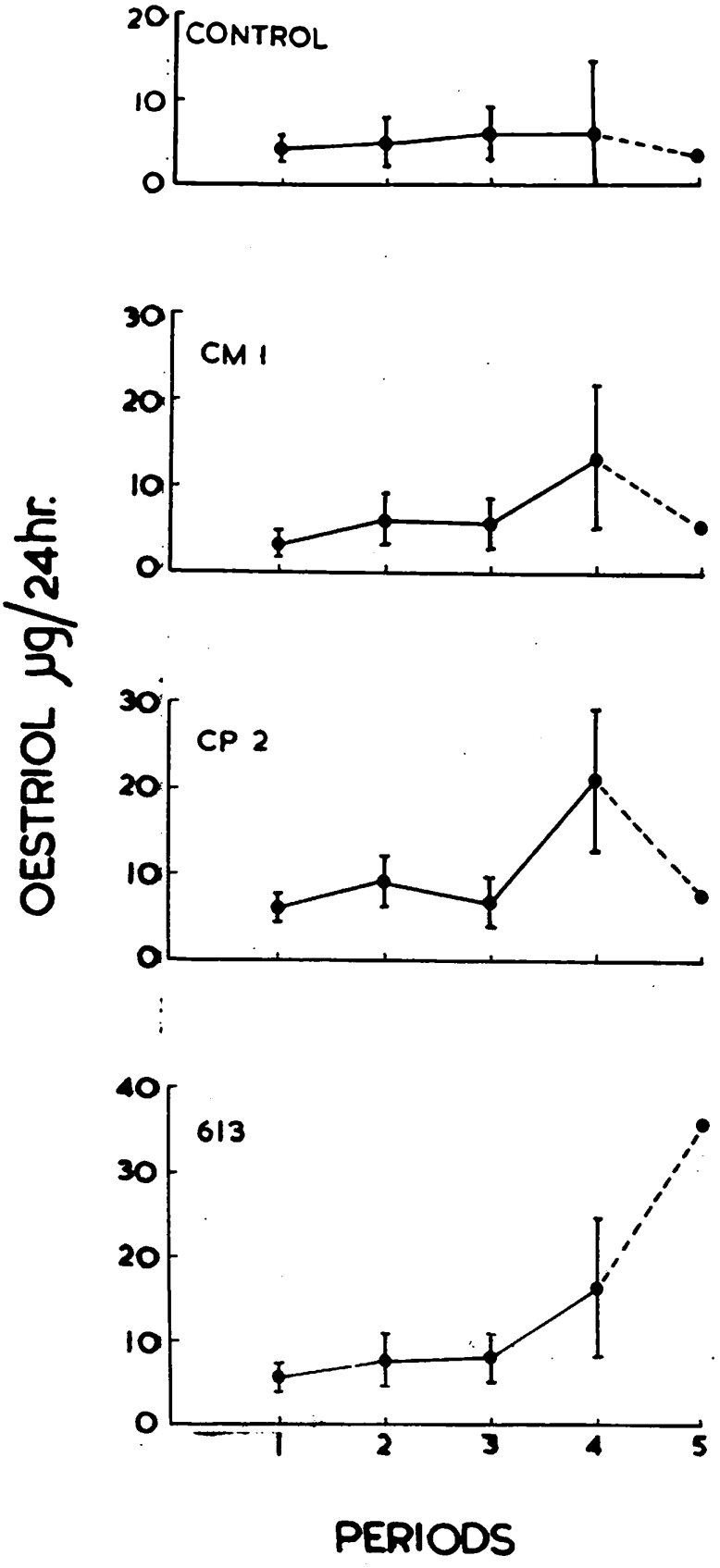
FIG. 5.



Average excretion of oestriol in periods 1 to 5 in the four patients

The bars represent the 95 per cent confidence limits in this and succeeding figures.

FIG. 6A



Effect of different treatments on the average excretion of oestriol

Table 1. Analysis of Variance.

Experiment 1: Oestriol.

Source	PERIOD 1 Sum of Squares	df	Mean Square	PERIOD 2 Sum of Squares	df	Mean Square	PERIOD 3 Sum of Squares	df	Mean Square	PERIOD 4. Sum of Squares	df	Mean Square
Between patients	680	3	227	5,061	3	1,687	4,878	3	1,626	93,094	3	31,031 **
Between treatments	3,830	3	1,277	8,671	3	2,890	2,794	3	931	98,928	3	32,976
Between order of treatments:												
Linear trend	2,616	1	2,616	1,601	1	1,601	58	1	58	1,306	1	1,306
Residual	12,352	2	6,176***	336	2	168	7,331	2	3,666	57,268	2	28,634
Residual/Square	3,853	6	642	8,810	6	1,468	3,795	6	632	53,756	6	8,959
Residual	6,805	16	425	17,456	12	1,455	23,693	16	1,481	197,043	16	12,315
Total	30,136	31		41,935	27		42,550	31		501,395	31	

In this and succeeding Tables:

. Significant at 10% level

* Significant at 5% level

** Significant at 1% level

*** Significant at 0.1% level

Table II Analysys of Variance

Experiment 1: Pregnanediol

Source	PERIOD 1			PERIOD 2			PERIOD 3			PERIOD 4			PERIOD 5		
	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square
Between patients	59.5	2	29.8	208	2	104	71	2	35.5	256	2	128	165	2	82.5
Between treatments	14.0	3	4.7	235	3	78.3	73	3	24.3	358	3	119.3	212	3	70.7
Interaction patients x treatments	237.5	6	39.6	453	6	75.5***	718	6	143.7***	336	6	56**	548	6	91.4
Residual	345	12	28.8	74	12	6.1	52	12	4.4	130	12	10.8	300	12	25
Total	656	23		970	23		914	23		1,080	23		1,225	23	

The experiment provided no evidence that the average excretion differed significantly from month to month.

The results of oestriol excretion for the four periods were combined into one analysys (Table III), which showed the following significant factors:

1. Period to period differences - the average for the fourth period was higher than that for the other three periods (Figure 7A).
2. Patient to patient differences - Patient A had a higher overall response than the other 3 patients (Figure 7B).
3. Treatment to treatment differences - CM 1, CP 2 and 613 produced a higher oestriol excretion than the control treatment. It is probable that CP 2 and 613 produced a higher response than CM 1, (Figure 7C).

Pregnanediol:

An analysys was made for each of the first five four-day periods. Patient C was found to have a significantly higher basal excretion than the others and therefore her figures were omitted from the analysis. The effects of different treatments for the other three patients are shown graphically in Figure 6B.

Periods 1 and 2: There were no significant differences between the average figures for different patients and for different preparations. Patient A showed a rise in excretion above the control level with CM 1, CP 2 and 613. Patient B showed no change with CM 1 or CP 2 but it is probable that there was a small increase with 613 while patient D showed a rise with CM 1 and CP 2 but not with 613.

Period 3: Patient A again showed a rise with 613 but with CM 1 and CP 2 her excretion did not differ from the control level. Patient B also probably showed a slight rise with 613 and no change with CM 1, or CP 2 but with patient D excretion rose with CM 1 and CP 2 and there was no evidence of a significant rise with 613.

Period 4: Patient A showed an appreciable rise above the control level with CP 2 and there was also a rise with CM 1 but with 613 it fell to the control level again. Patient B showed no change with CM 1 or CP 2 but it is probable that there was a small increase above the control level with 613. In patient D excretion remained above the control level with CM 1 and CP 2 but there was no evidence of a significant rise with 613.

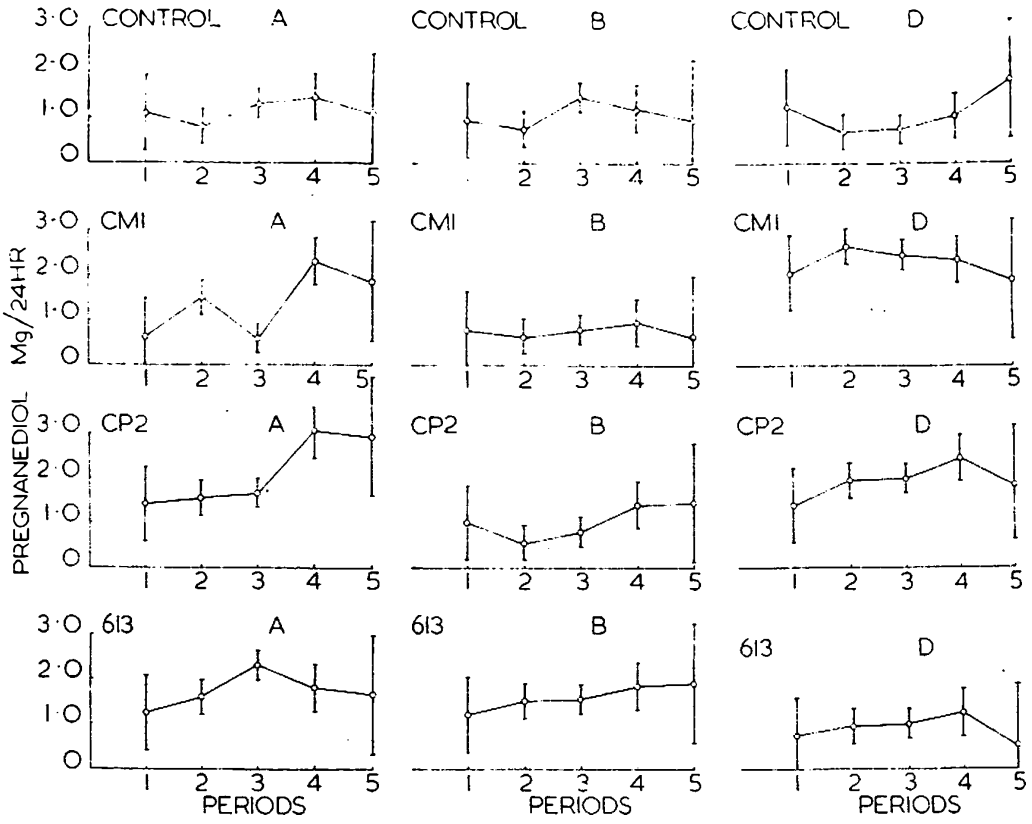
Table III Analysis of Variance

Experiment 1: Oestriol

Source	Sum of squares	df	Mean square
Between periods	162,669	3	54,223***
Between patients	50,293	3	16,764***
Between treatments	59,609	3	19,870***
Interactions:			
Periods x patients	53,421	9	5,936
Periods x treatments	54,615	9	6,068
Patients x treatments	36,184	9	4,020
Periods x patients x treatments	116,898	27	4,330
Residual	244,997	60	4,083
Total	778,686	123	

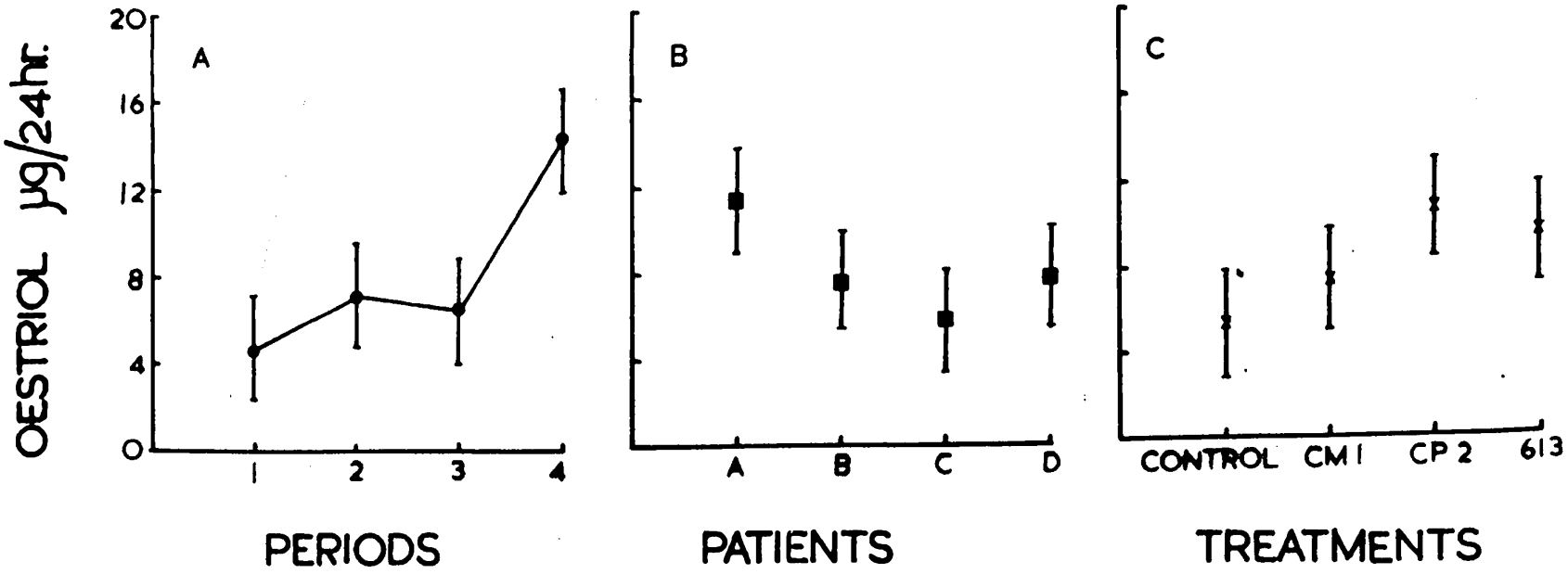
*** Significant at 0.1% level.

FIG. 6B.



Effect of different treatments on the excretion of pregnanediol by patients A, B and D.

FIG. 7



Average excretion of oestriol for each period

Average excretion of oestriol for each patient

Average excretion of oestriol for the four patients with different treatments

Period 5: As in period 1 the apparent differences could be readily explained by chance variation.

Combining the results of pregnanediol excretion for the 5 periods into one analysis confirmed the above observations without providing any further information.

Conclusions:

The patients showed different degrees of sensitivity to treatment suggesting that they require different doses. The graphical summary of the results, shown in Figures 5 and 6, suggests that CP 2 had produced the most normal pattern of excretion of oestriol and pregnanediol and that the response with CM 1 and 613 was less than with CP 2. There was no evidence of any difference in response in different months of treatment.

Experiment 1A.
Treatment with (CP 2), the gonadotrophin preparation that appeared to give the best responses in Experiment 1.

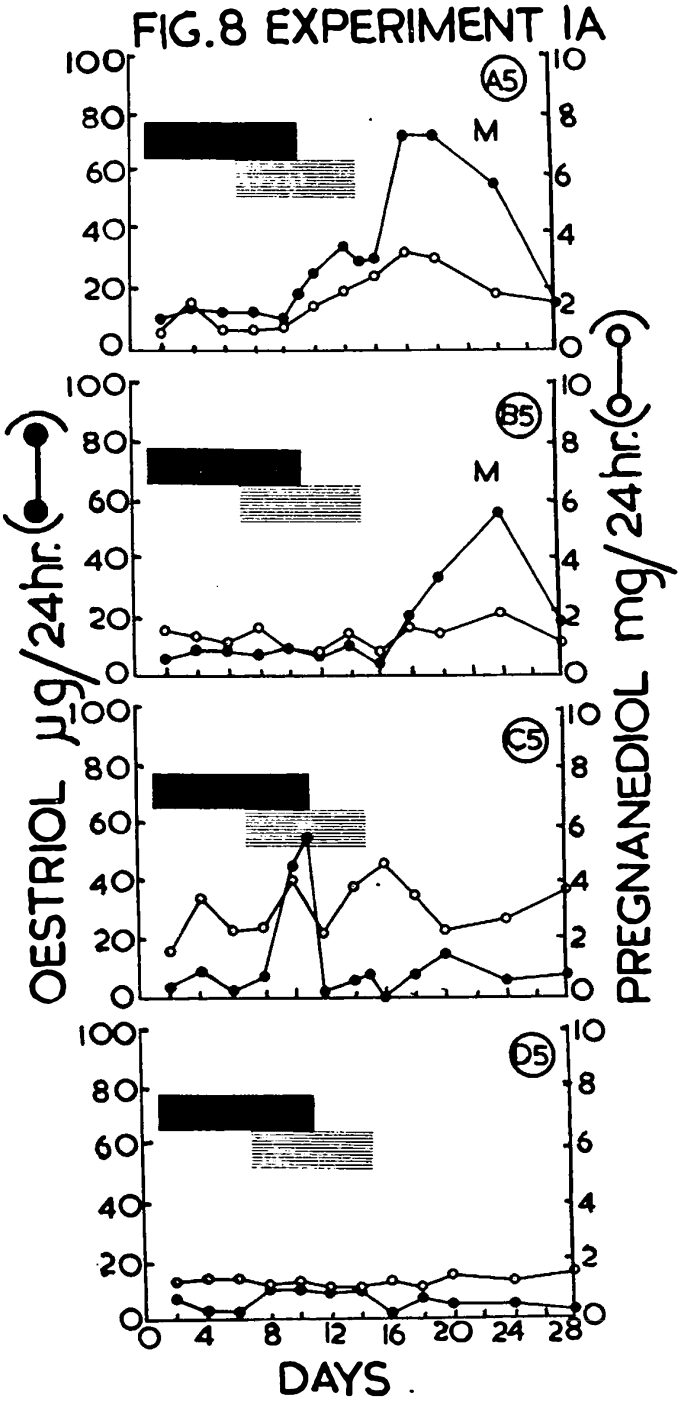
Since Experiment 1 had shown that CP 2 gave the most promising results this material was given in the same dosage and for the same number of days to all four patients again. In order to try to increase the responses, however, particularly in patients C and D, they were all given H.C.G. in a dosage of 6,000 i.u. daily for eight days instead of four, from the 7th to the 14th day inclusive. Thus they received HCG simultaneously with F.S.H. for four days, from the 7th to the 10th day inclusive.

This short experiment was not analysed statistically since the differences between the responses of the patients were obvious from the plotted results.

The effect of increasing the total dosage and the length of treatment with H.C.G. was surprising. Patient A showed a similar rise in excretion of oestriol at mid-cycle and of pregnanediol in the luteal phase (Figure 8, A5) to that seen previously with CP 2 (Figure 4, A3). The rise in oestriol however was followed by a sustained secondary rise during the luteal phase. This was followed by menstruation. Patient B showed only a sustained late rise in excretion of oestriol without any appreciable change in the excretion of pregnanediol (Figure 8, B5). This was also followed by menstruation. Patient C showed an early rise in excretion of oestriol but no change in pregnanediol (Figure 8, C5) and patient D failed to respond (Figure 8, D5). Neither patient C nor D menstruated.

All four patients were readmitted for a second examination under anaesthesia and culdoscopy on the 15th day of the cycle. The results are shown in Table IIIA. The uterus and ovaries of patient A had increased in size and she showed developing follicles but no corpora lutea. The uterus of patient B was larger than when first examined while those of patient C were unchanged. The uterus of patient D was slightly larger but her ovaries were unchanged.

The stroma and glands of the endometrium of patients A and B now looked functional and in the proliferative phase whereas those of patient C showed very little change and of D no change.



The excretion of oestriol and pregnanediol by patients A, B, C and D, given FSH (CP 2) and HCG.

TABLE III A

Clinical Data Relating to Patients After Experiment 1A

Patients	A	B	C	D
Ovaries, size (cm.)	2.5 x 2.5	2.5 x 2	2.0 x 0.5	1.8 x 1.8
Ovaries, cysts	Follicles present	1 ? ruptured follicle	Nil	Nil
Uterus, sound (cm.)	8.7	6.25	6.25	6.25
Endometrium	Functional Proliferative	Functional Proliferative	Scanty, slight Proliferative	Scanty inactive

Conclusions:

None of the patients had a response which was similar to the normal pattern. The response of patient A was nearest to normal and the varying responses confirmed the suggestion in the first experiment that the patients would require different dosage levels. The observations made by culdoscopy further confirmed the differences in response between patients.

After Experiment 1A the patients had two months with no treatment to establish whether they would now menstruate spontaneously, but none did.

Experiment 2.

The effect of varying the total and daily dosage of F.S.H. on steroid production.

This experiment was designed to test the effect of varying (a) the total dosage and (b) the daily dosage of F.S.H. The preparation used throughout the experiment was CP 1 as no more CP 2 was available.

Since it had been shown previously that there was a difference in sensitivity of the different patients to F.S.H., and since pregnancy was the primary objective of the experiment, it was decided to vary the dosage arbitrarily to suit each patient. The dosages for each patient were therefore called low (L), medium low (ML), medium high (MH), and high (H). Since it had also been shown previously that the order of treatment did not appear to alter its effects it was decided to give each patient the ML dose in the first month. If at the end of the first month it was found that this dosage was too great or too small for any of the patients it was still possible to adjust it for the individual patient concerned by calling this dose H, MH, or L instead of ML, and this was found in practice to be necessary for patients A and D.

It was decided to reduce the treatment with F.S.H. arbitrarily from ten days to eight days and to divide the total dosage of CP 1 so that it was reduced by a factor of 0.9 each day in one month, 0.8 in another and 0.7 in a third, while in the fourth month the daily dosage would remain constant so that the factor was 1.0 These factors have been called the rates of fall-off. The final total dosages and the factors varying the daily dosages are shown in Table IV.A

TABLE IVA

RATE OF FALL OFF OF F.S.H.

PATIENT	0.7	0.8	0.9	1.0
A	2400 L	2640 ML	2904 MH	3200 H
B	3200 MH	2640 L	3520 H	2904 ML
C	4184 H	3800 MH	3456 ML	3128 L
D	4120 ML	4981 H	3744 L	4528 MH

Table shows the Total dose of F.S.H. (CP 1) in

terms of mg. equivalents IRP-HMG used with different rates of fall off. The letters designate the level of dose used in each patient.

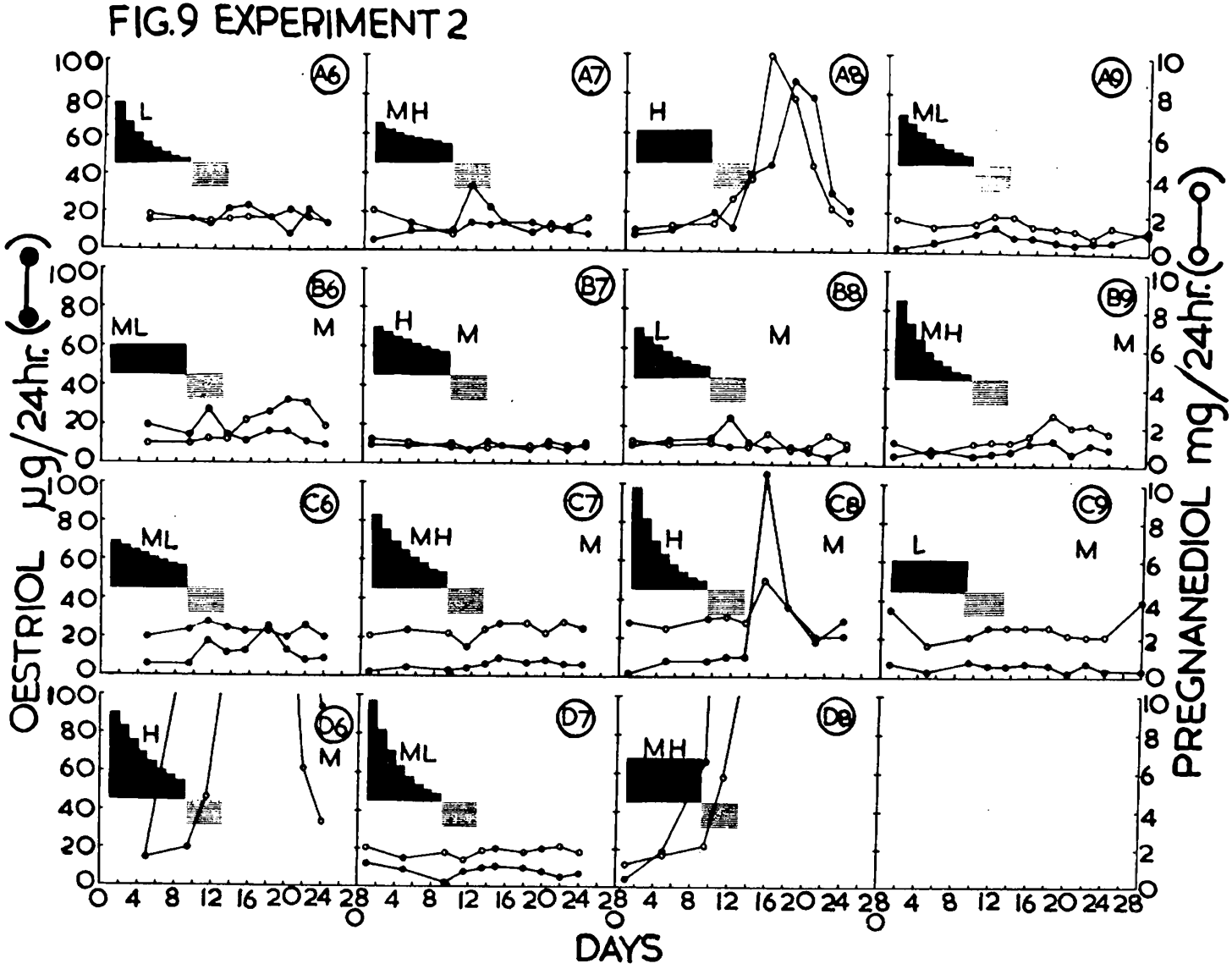
H.C.G. (6,000 i.u.) was given daily for four days as in Experiment 1.

Perusal of the data obtained so far showed that the peak in excretion of oestriol at mid-cycle was of short duration and it was possible that small peaks would be missed if the urine was examined only on alternate days. Two day collections of urine were therefore pooled over a period of eight days. These pooled samples were for days 8 and 9, 10 and 11, 12 and 13, and 14 and 15. It was decided to examine also isolated samples on days 4, 17, 19, 21, 23 and 28, the last serving as control for the following month. The normal patterns of excretion of oestriol and pregnanediol based on these samples was given in Figures 2B and 3C.

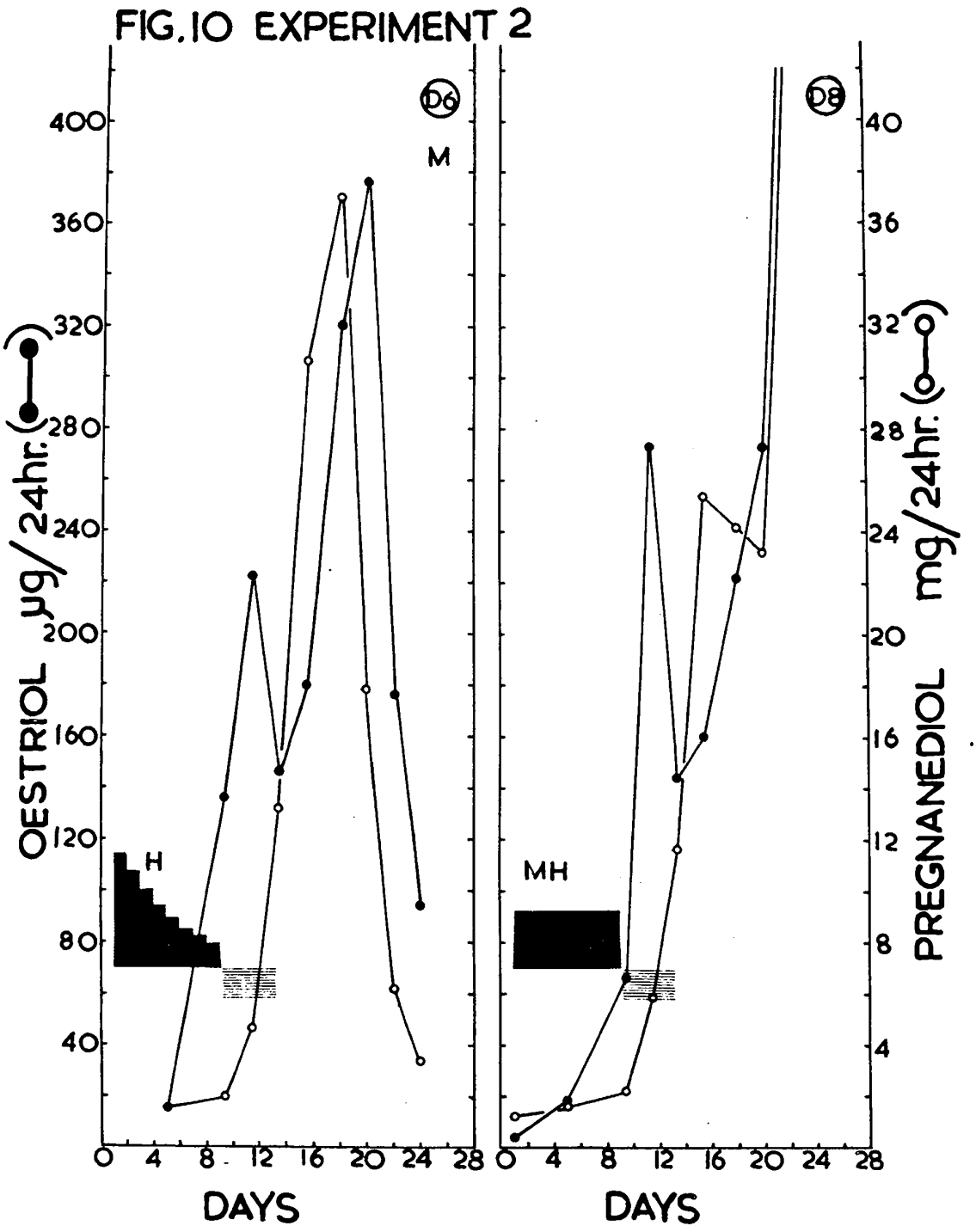
The results and dosages are shown graphically in Figure 9. Patient A showed a doubtful rise in excretion of oestriol on L dose at a rate of fall-off of 0.7 (Figure 9, A6), a good rise on MH at 0.9 (Figure 9, A7) and no rise on ML at 0.8 (Figure 9, A9) but there was no rise in pregnanediol in any of these months. On H at 1.0 (Figure 9, A8), however, she showed a slight rise in excretion of oestriol on days 8 and 9 followed by a fall and then a pronounced rise in the luteal phase. There was also a pronounced rise in excretion of pregnanediol which reached a maximum level of 9.9mg/24hr. during days 14 and 15. She failed to menstruate after any of these treatments.

Patient B showed a normal pattern of excretion of oestriol and of pregnanediol on ML at 1.0 and menstruated scantily afterwards (Figure 9, B6). She showed no response on H at 0.9 (Figure 9, B7) and a slight rise of oestriol at mid-cycle on L at 0.8 (Figure 9, B8). Both were followed by slight bleeding. There was no change in excretion of oestriol on MH at 0.7 (Figure 9, B9) but surprisingly there was an increase in pregnanediol during the luteal phase and she menstruated.

Patient C showed a slight rise in excretion of oestriol at mid-cycle and another during the luteal phase on ML at 0.9 (Figure 9, C6) but no response on MH at 0.8 (Figure 9, C7). She had a scanty menstruation after the latter. There was a striking



The excretion of oestriol and pregnanediol by patients A, B, C and D given different total and daily doses of FSH (CP 1) and HCG.



The excretion of oestriol and pregnanediol by patient D given different total and daily doses of FSH (CP 1) and HCG.

rise in oestriol but only a slight rise in pregnanediol in the luteal phase on H at 0.7 (Figure 9, C8) followed by menstruation. There was no rise on L at 1.0 (Figure 9, C9) but she had another scanty menstruation. Changes in excretion of pregnanediol were obscured by her high control figures.

Patient D showed a striking rise of oestriol on days 10 and 11 on H at 0.8 (Figures 8 and 10, D6). This was followed by a fall on days 12 and 13 and then it soared to 376 µg./24hr. by the 17th day. Both steroids fell to normal again on the 28th day and she menstruated for the first time. On ML at 0.7 she failed to respond (Figure 9, D7). On MH at 1.0 the excretion of both oestriol and pregnanediol showed changes which were remarkably similar in quantity and timing to those observed previously on H at 0.8 (Figures 9 and 10, D8). On this occasion, however, they continued to rise progressively, oestriol reaching a figure of 856 µg./24hr. on the 28th day and pregnanediol 78.5mg/24hr. on the 23rd day of the cycle suggesting pregnancy. This was subsequently confirmed.

Analysis of Results:

The 28 day cycle was divided into five periods each containing two observations. These periods were:- (1) days 1-4, (2) days 9-10, (3) days 13-16, (4) days 17-20, (5) days 21-24. No observations were made during days 5-8 or days 25-28. The effects of treatment were analysed separately for oestriol and pregnanediol as in Experiment 1.

Because of the design of the experiment it was possible to isolate and examine separately the effects of three factors; the differences between patients, between different dosages of F.S.H. and between the different rates of fall-off in dosage. It was originally designed as a Latin Square in which each patient was given each of the four dosage levels of F.S.H. using each of the four rates of fall-off in dosage. However, the results for patient D who became pregnant during the third month of the experiment had to be omitted leaving the rest in the pattern of a Youden square. Each period was analysed separately for oestriol and for pregnanediol as shown in Tables IV and V.

Oestriol:

The apparent differences between the average excretion of oestriol in different patients and following

Table 1V Analysis of Variance.

Experiment 2: Oestriol.

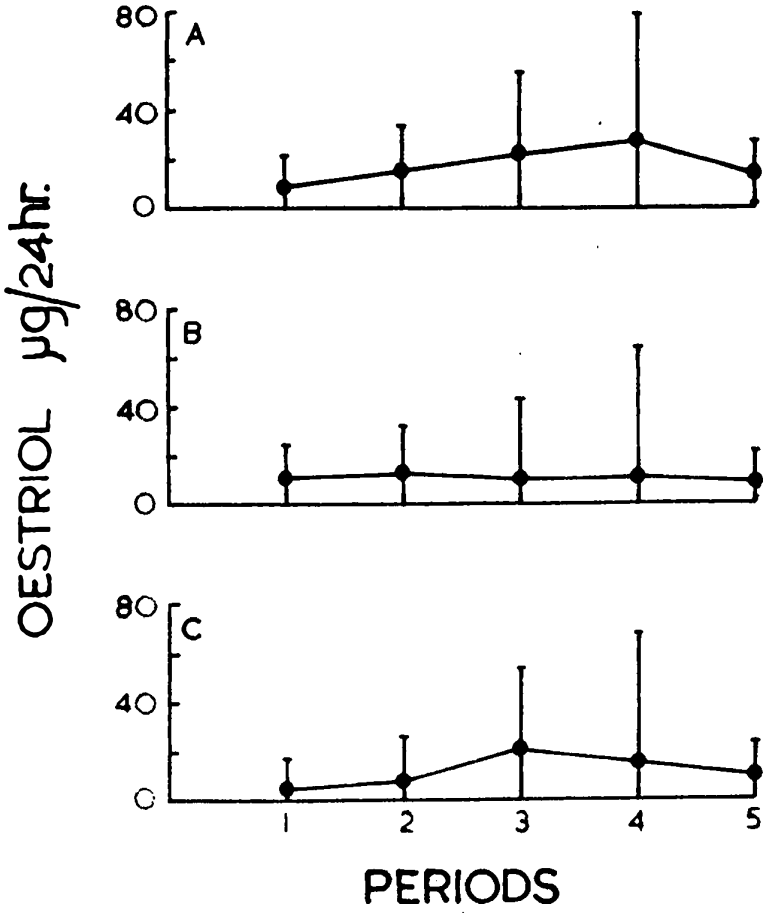
Source	PERIOD 1			PERIOD 2			PERIOD 3			PERIOD 4			PERIOD 5		
	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square
Between levels (adjusted)	64	3	21	63	3	21	2,149	3	716	3,348	3	1,116	274	3	91
Between fall-off (ignoring levels)	91	3	-	92	3	-	1,504	3	-	2,439	3	-	356	3	-
Between fall-off (adjusted)	79	3	26.3	75	3	25	918	3	306	1,795	3	598	214	3	71
Between levels (ignoring fall-off)	76	3	-	80	3	-	2,735	3	-	3,992	3	-	416	3	-
Between patients	179	2	90	256	2	128	706	2	352	1,270	2	635	146	2	73
Residual/square	167	3	56**	341	3	114	1,139	3	380	2,944	3	981***	166	3	55.3**
Residual	56	9	6.2	564	12	47	4,503	12	375	303	12	25.2	96	12	80
Total	557	20		1,316	23		10,001	23		10, 304	23		1,038	23	

Table V Analysis of Variance

Experiment 2: Pregnanediol

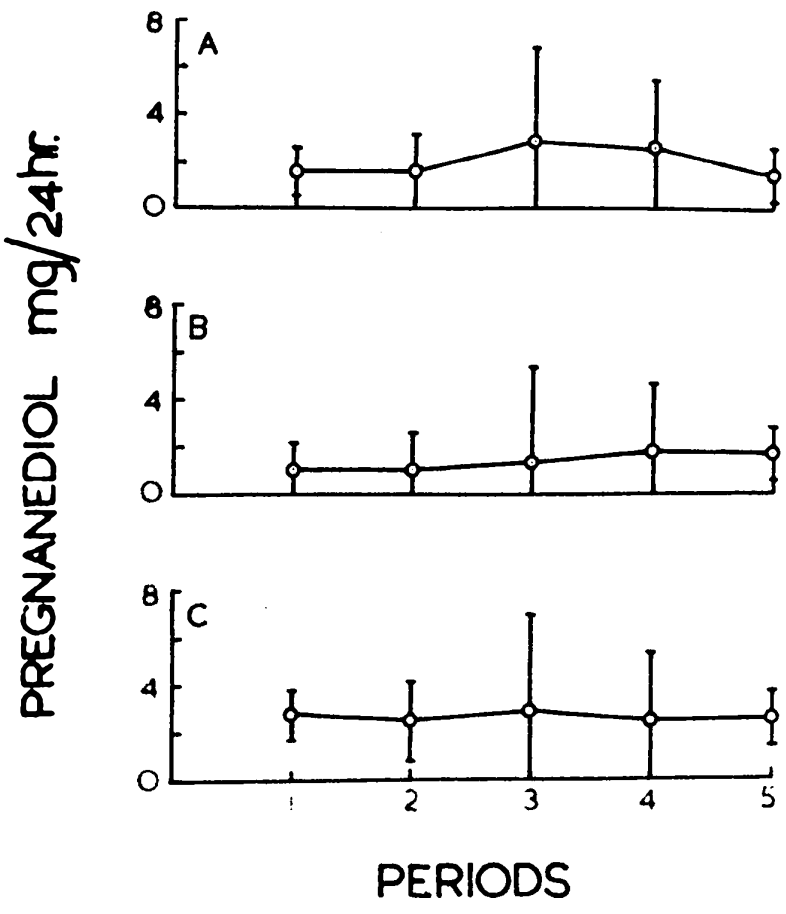
Source	PERIOD 1			PERIOD 2			PERIOD 3			PERIOD 4			PERIOD 5		
	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square
Between levels (adjusted)	0.29	3	0.10	1.13	3	0.38	17.51	3	5.84	8.59	3	2.86	1.29	3	0.43
Between fall-off (ignoring levels)	0.12	3	-	0.87	3	-	16.66	3	-	21.65	3	-	1.65	3	-
Between fall-off (adjusted)	0.08	3	0.03	0.87	3	0.29	15.78	3	5.26	22.28	3	7.43	2.50	3	0.83*
Between levels (ignoring fall-off)	0.33	3	-	1.13	3	-	18.39	3	-	7.96	3	-	0.44	3	-
Between patients	7.26	2	3.63**	8.65	2	4.32	13.23	2	6.62	3.43	2	1.72	4.60	2	2.30**
Residual/square	1.09	3	0.36	2.63	3	0.88**	10.95	3	3.65	8.91	3	2.97*	1.34	3	0.45
Residual	6.26	12	0.52	1.61	12	0.13	22.63	12	1.89	7.82	12	0.65	1.78	12	0.15
Total	14.61	23		12.89	23		80.98	23		50.40	23		10.66	23	

FIG. 11A



Average excretion of oestriol in periods 1 - 5 in patients A, B and C.

FIG. 11B



Average excretion of pregnanediol in periods 1 - 5 in patients A, B and C.

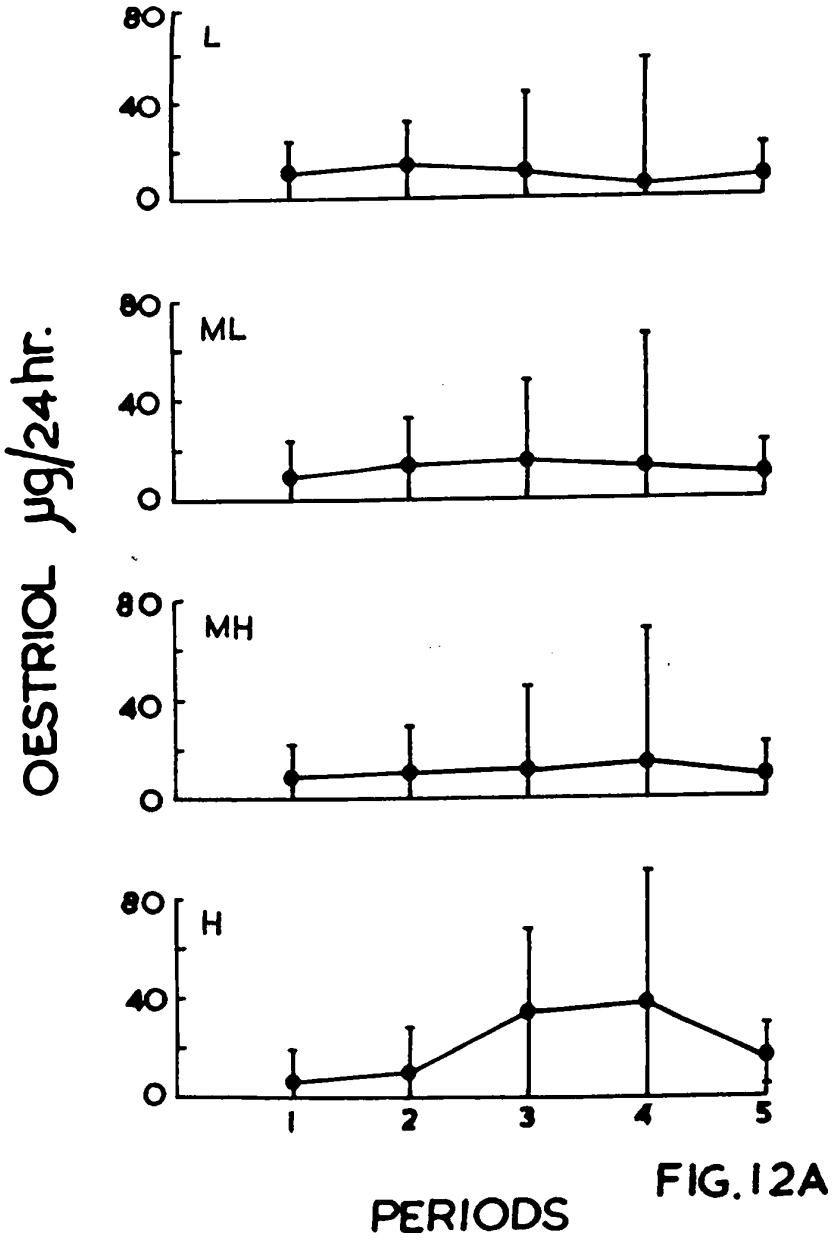


FIG. 12A

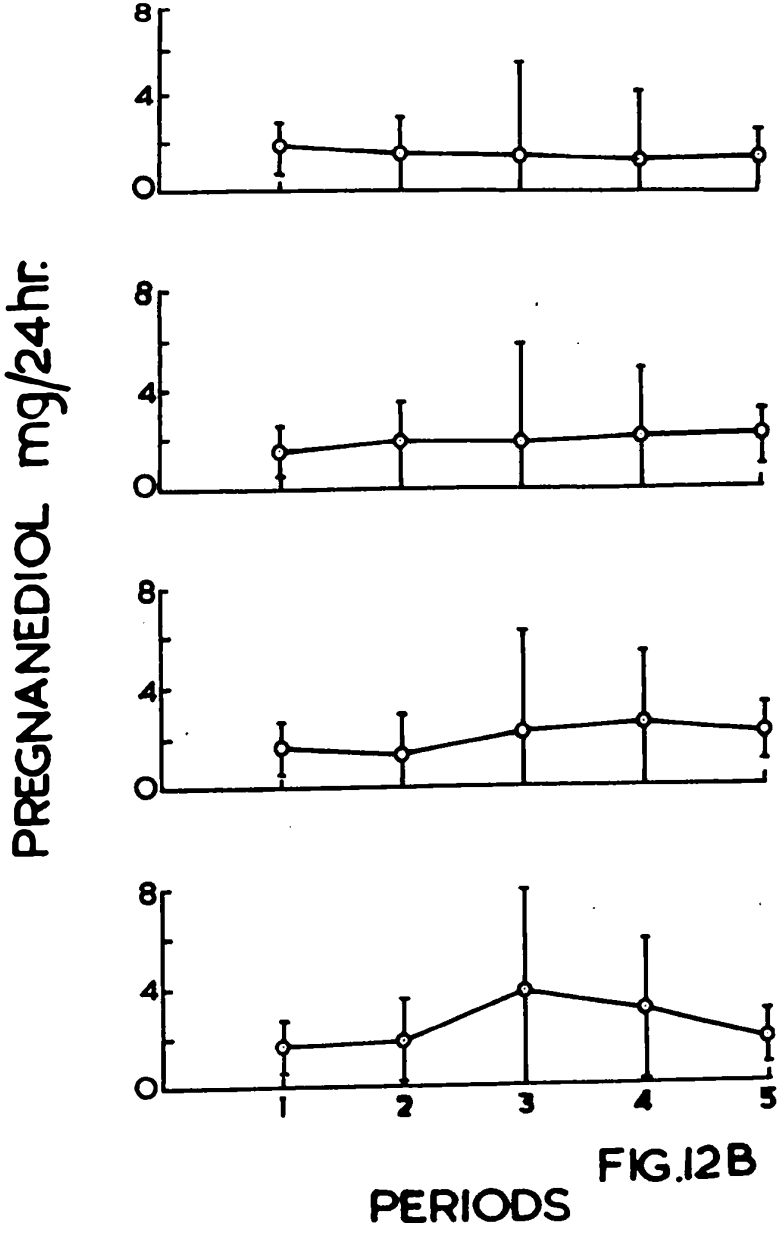


FIG. 12B

Fig. 12A.

Effect of different dosages of FSH on the average excretion of oestriol for patients A, B and C.

Fig. 12B.

Effect of different dosages of FSH on the average excretion of pregnanediol for patients A, B and C.

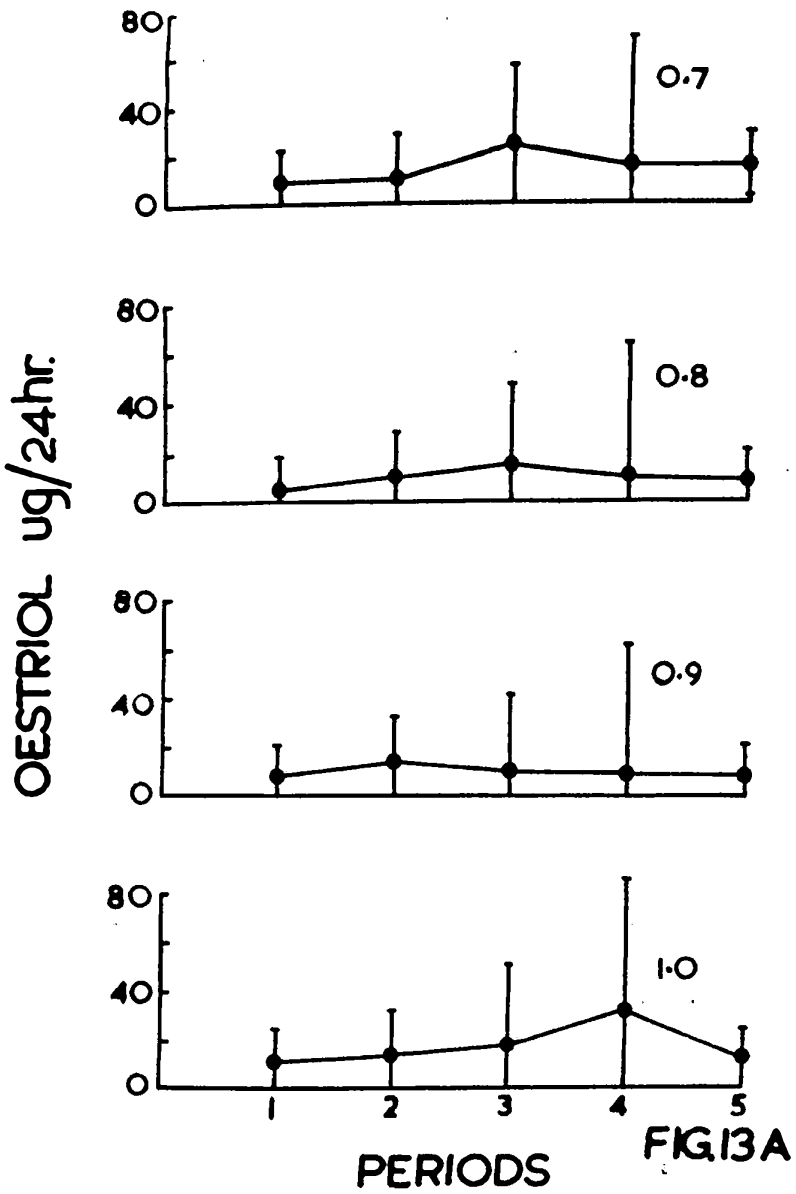


FIG.13A

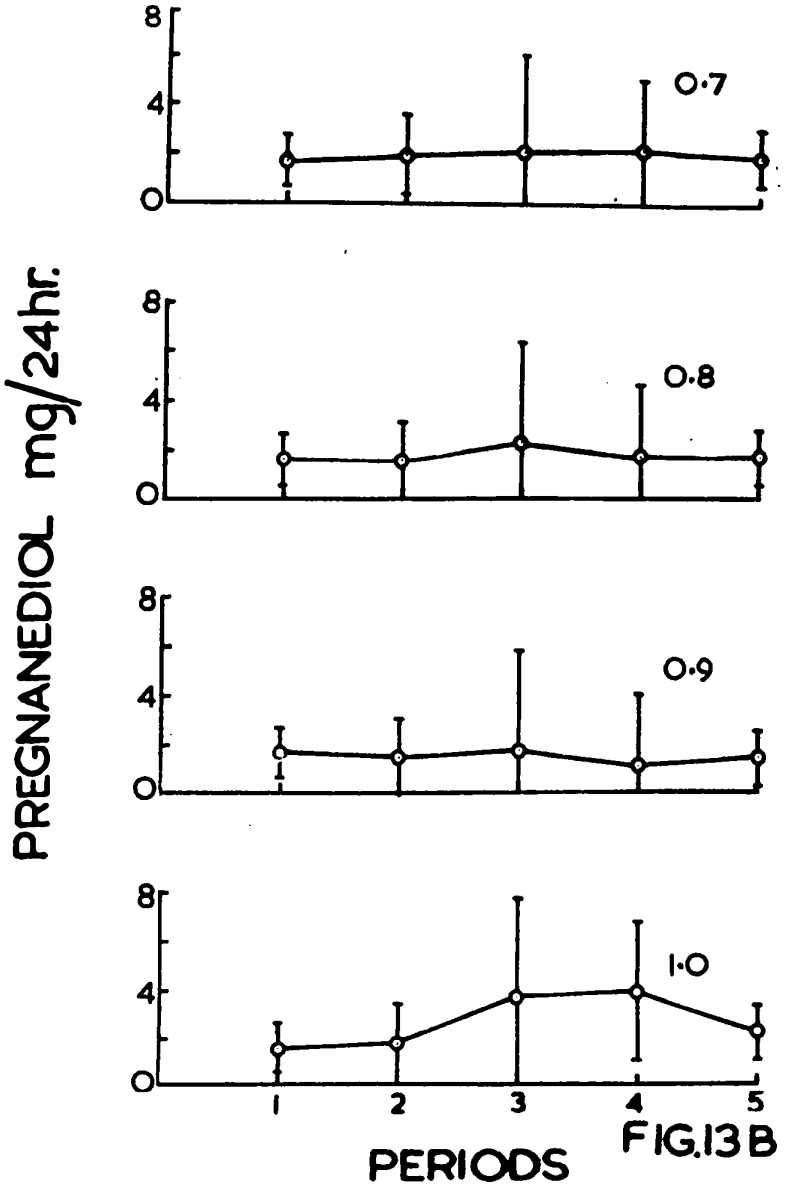


FIG.13B

Fig. 13A.

Effect of different rates of fall-off on the average excretion of oestriol for patients A, B and C.

Fig. 13B.

Effect of different rates of fall-off on the average excretion of pregnanediol for patients A, B and C.

different dosages of F.S.H. and different rates of fall-off in dosage during each of the periods could easily have occurred by chance. (Figures 11, 12 and 13A and Table IV).

Pregnanediol:

The differences between patients was only significant during periods 2 and 5 and this was due to the high basal level of patient C (Figure 11B and Table V). There was not sufficient evidence that the apparently large differences in the effect of different dosages of F.S.H. were not due to chance (Figure 12B). The differences between the different rates of fall-off could also readily be attributed to chance except in period 5 (Figure 13B) in which treatment without fall-off produced a higher level of pregnanediol excretion than treatment with fall-off.

Conclusions:

This experiment indicated that the dosage levels chosen for the three patients were in the correct proportion. Although the differences were not significant the results in Figures 12A and B suggest that the response to the high dosage was better than to the other dosages and the results in Figures 13A and B that the response to treatment at a constant daily dosage was slightly better than with any of the rates of fall-off. In subsequent experiments therefore a higher dosage at a constant level was adopted, and the relative levels for the different patients were maintained.

No treatment was given in the month after this experiment was completed and none of the three remaining patients menstruated spontaneously.

Experiment 3:

The effect of varying the total and daily dose of H.C.G. used with two levels of F.S.H.

In this experiment patient E was substituted for patient D who had become pregnant. The experiment was designed primarily to test varying methods of treatment with chorionic gonadotrophin. Four doses of H.C.G. were used, 3,000, 6,000, 12,000 and 24,000 i.u. They were arranged so that the whole dose was given in a single injection in one instance and divided into four equal daily doses in another. The single injection was given with the last dose of F.S.H. or on the following day; the four injections were given simultaneously with the last four doses, or two with and two after the last doses of F.S.H. In addition two dosages of F.S.H. were used for each patient, high (H) and low (L), with a dose interval of 1.33, the individual dosages varying with patients A, B and C on the basis of their previous responses. An intermediate pair of dosages were chosen for patient E.

The individual dosages are shown in the Table.

The treatment for each patient was derived from the combination of factors being investigated as shown in table below.

Order of Treatments.	Patients: A	B	C	E
4th month	a	b	c	e
3rd month	d	cde	bde	ade
1st month	bce	ace	abe	abc
2nd month	abcde	abd	acd	bcd

The factors corresponding to each of the code letters were:-

H.C.G. to overlap (a) or not overlap (-) F.S.H.

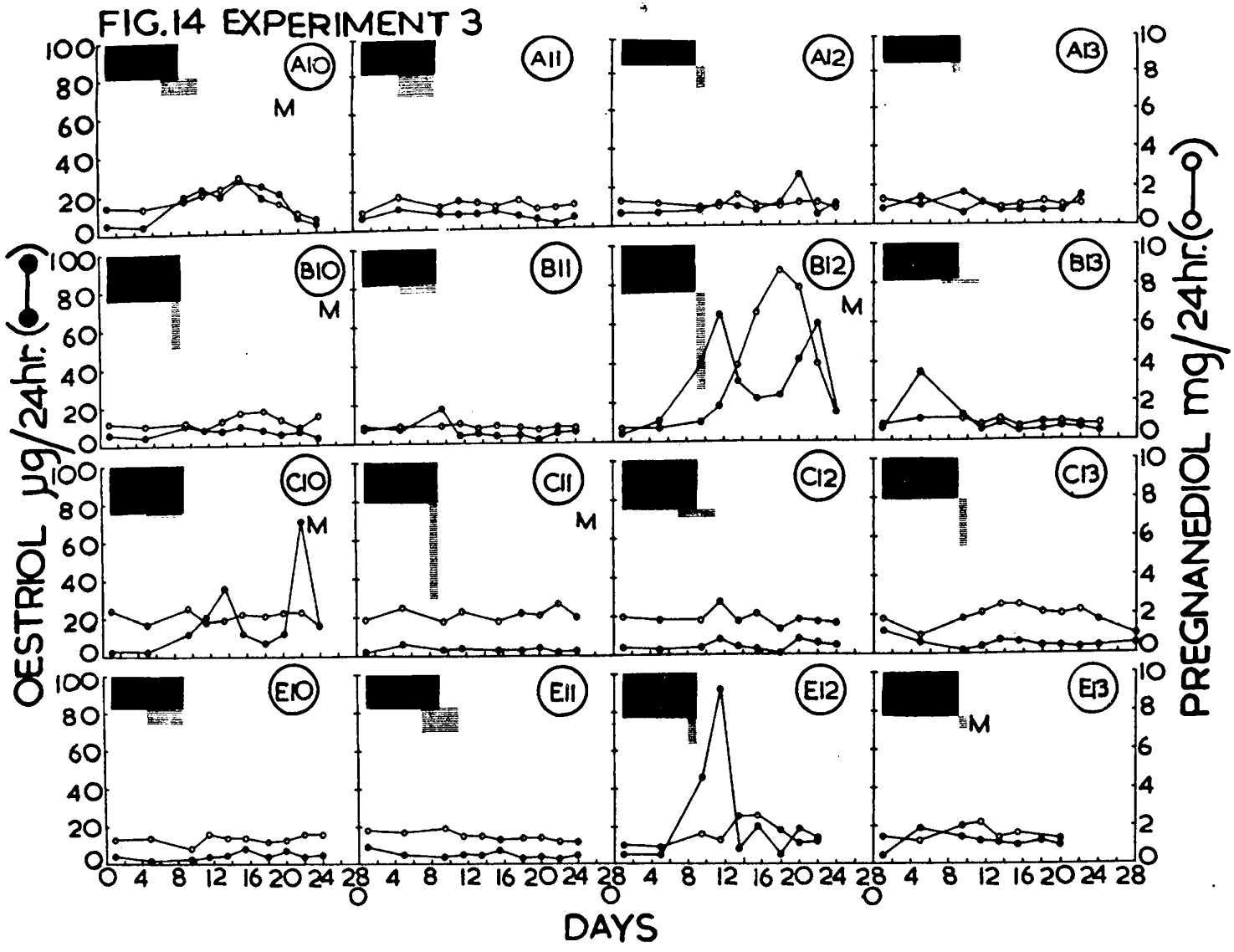
H.C.G. to be given in 1 or 4 injections (b).

Total dose of H.C.G. to be

3,000 - -; 6,000 (d), 12,000 (c), 24,000 (cd) i.u.

The levels of F.S.H. adjusted for each patient (0), (e).

The excretion of oestriol and pregnanediol were measured in urine which was collected and pooled as in Experiment 2. The results and dosages are shown graphically in Figure 14.



The excretion of oestriol and pregnanediol by patients A, B, C and E given different dosages of FSH.

HCG was given in different total dosages in one or four injections given with or partly or wholly after FSH.

Two levels of F.S.H. were used as indicated by the large and small dense black area on each months treatment, the parallel lines represent (a) the total dose of H.C.G. used (b) the way in which it was given - either in 1 or 4 injections (c) whether given with or after F.S.H.

Patient A given H dosage of F.S.H. and 12,000 i.u. H.C.G. in four doses with partial overlap showed a rise in excretion of oestriol on days 10 and 11 followed by a fall and later a secondary rise together with a rise of pregnanediol in the luteal phase (Figure 14, A10). She subsequently menstruated. On H dosage with 24,000 i.u. H.C.G. in four doses and complete overlap, however, she showed no response (Figure 14, A11). On L dosage with 6,000 i.u. H.C.G. in a single dose and no overlap she showed only a late rise in excretion of oestriol in the luteal phase (Figure 14, A12) and on L dosage with 3,000 i.u. H.C.G. in a single dose with overlap she showed no response (Figure 14, A13).

Patient B given H dosage and 12,000 i.u. H.C.G. in a single dose with overlap showed only a doubtful rise of pregnanediol in the luteal phase with no rise of oestriol and she menstruated (Figure 14, B10). On L dosage and 6,000 i.u. H.C.G. in four doses and complete overlap she showed a slight rise in excretion of oestriol on days 8 and 9 (Figure 14, B11). On H dosage and 24,000 i.u. H.C.G. in a single dose without overlap she showed a pronounced rise of oestriol on days 10 and 11. This was followed by a considerable fall and a second rise of similar magnitude later in the cycle. The pregnanediol rose to a high level between these two peaks of oestriol excretion but both returned to normal at the end of the cycle and she menstruated (Figure 14, B12). On L dosage and 3,000 i.u. H.C.G. in four doses with partial overlap she showed a considerable rise in excretion of oestriol on the 4th day of treatment with F.S.H. but it returned to control levels subsequently and there was no rise when changes are normally expected to occur. She failed to menstruate (Figure 14, B13).

Patient C given H dosage and 3,000 i.u. H.C.G. in four doses with overlap showed a good rise in excretion of oestriol on days 12 and 13 followed by a return to the control level and a second good rise in the luteal phase. There was no change in excretion of pregnanediol

but she subsequently menstruated (Figure 14, C10). In the next three months she failed to respond to any treatment (Figure 14, C11, 12 and 13) except for a scanty period after the first of these.

Patient E was give L dosage for the first two months and failed to respond (Figure 14, E10 and 11). On H dosage with 6,000 i.u. H.C.G. in a single dose with overlap, however, she showed a pronounced rise in excretion of oestriol followed by some further fluctuations. There was a moderate early but ill-sustained rise of pregnanediol and she failed to menstruate (Figure 14, E12). On H dosage with 3,000 i.u. H.C.G. in a single dose without overlap she showed a slight rise in excretion of oestriol on the fourth day of treatment followed by a slight and early rise of pregnanediol occurring on days 8 to 11. She menstruated correspondingly early (Figure 14, E13).

Analysis of Results.

The experimental design permitted the isolation and separate estimation of the following:- the avergae effect of differences between patients, between different dosages of H.C.G. and between different dosages of F.S.H. of differences in the timing of treatment with H.C.G; of differences in the number of injections of H.C.G. and finally the extent to which some of these factors were interdependent. For example, it was found that the effect of different dosages of H.C.G. depended on the timing of the injections, the number of injections and also the dosage of F.S.H. The statistical analysis allowed the interdependence of the dosage of H.C.G. and its timing to be isolated free from the bias of other significant effects. In a similar manner, using all the experimental data once again, the interdependence of the dosage and the number of injections of H.C.G. was estimated free from the bias of the other significant effects. Finally, it was possible to estimate the interdependence of the dosage of H.C.G. and the dosage of F.S.H. in the same manner.

There were four dosage levels of H.C.G. in this experiment, and the analysis allowed the effects of two alternative pairs of higher and lower dosages to be assessed. In the first groupings the higher levels were 12,000 and 24,000 i.u. with an avergae dosage of 18,000 i.u., and the lower levels were 3,000 and 6,000 i,u. with an average dosage of 4,500 i.u. In the second

groupings, the higher levels were 6,000 and 24,000 i.u. with an average dosage of 15,000 i.u., and the lower levels were 3,000 and 12,000 i.u. with an average dosage of 7,500 i.u. Since only a half of the complete factorial design was used, the interaction of the dosage of H.C.G. with the other factors had to be assessed by using one or other of these alternative methods of grouping.

The cycle of 28 days was divided into five periods each containing two observations exactly as in Experiment 2. Each period was analysed separately for oestriol and pregnanediol as before. (Table V1 and V11).

Oestriol:

Period 1. There was no significant difference in excretion between the four patients and no evidence that any treatment affected it. (Figure 15A to 18A).

Period 2. Patients B and E had a higher average response to treatment than patient A who in turn had a higher average response than patient C (Figure 15A).

The effect of an increased dosage of H.C.G. was dependent on both the timing and the number of injections. The two highest dosages (average 18,000 i.u.) given partly or wholly after F.S.H. produced a higher response than when given simultaneously with it. On the contrary the lower dosages (average 4,500 i.u.)given simultaneously with F.S.H. produced a higher response than when given partly or wholly after it (Figure 16A). The higher dosages (average 15,000 i.u.) given in a single injection produced a higher response than all the other alternatives (Figure 17A ii) and finally the higher dosages of H.C.G (average 15,000 i.u.) given with the high dosage of F.S.H. also produced a higher response than all the other alternatives (Figure 18A iv).

Period 3. The effect of an increased dosage of H.C.G. was dependent on the timing and the number of injections. This interdependence followed the same pattern as in period 2 (Figures 16A and 17A).

The higher dosages of E.S.H. produced a higher average excretion than the lower dosages (Figure 18A).

Period 4. The average response was almost identical to that in Period 3. The only difference was in the interdependence of dosage and timing of H.G.G, The higher dosages (average 18,000 i.u.) given wholly or partly after F.S.H. gave a higher response than the other alternatives (Figures 16A ii).

Table VI Analysis of Variance

Experiment 3: Oestriol.

Source	PERIOD 1			PERIOD 2			PERIOD 3			PERIOD 4			PERIOD 5		
	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square
Overlap	66	1	66	17	1	17	28	1	28	294	1	294	50	1	50
Number of injections	1	1	1	1,001	1	1,001	1	1	1	166	1	166	18	1	18
Dose of FSH	55	1	55	2,538	1	2,538	630	1	630**	371	1	371*	613	1	613
Dose of HCG	194	3	65	1,019	3	340	84	3	28	43	3	14	643	3	214
Patients	134	3	45	1,257	3	419*	51	3	17	327	3	109*	316	3	105
Interactions	156	6	26												
Overlap x no. of injections				23	1	23	72	1	72	81	1	81	378	1	378
Overlap x dose of HCG (1)				1,906	1	1,906**	392	1	392**	132	1	132*	946	1	946*
Overlap x dose of HCG (2)				87	1	87	29	1	29	58	1	58	800	1	800*
Overlap x dose of FSH				14	1	14	1	1	1	69	1	69	6	1	6
No. of injections x dose of HCG				1,815	1	1,815**	407	1	407**	505	1	505**	648	1	648
Dose of FSH x dose of HCG				639	1	639*	16	1	16	0	1	0	12	1	12
Residual	596	16	37.2	1,589	16	99.3	462	16	29	431	16	27	2,503	16	156
Total	1,202	31		11,906	31		2,170	31		2,170	31		6,934	31	

Table VII Analysis of Variance

Experiment 3: Pregnanediol

Source	PERIOD 1			PERIOD 2			PERIOD 3			PERIOD 4			PERIOD 5		
	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square	Sum of squares	df	Mean square
Overlap	2	1	2	36	1	36	313	1	313	1	1	1	0	1	0
Number of injections	23	1	23	0	1	0	313	1	313	41	1	41	98	1	98
Dose of FSH	2	1	2	72	1	72*	704	1	704	66	1	66**	15	1	15
Dose of HCG	29	3	10	28	3	9	522	3	174	126	3	42	164	1	164* (linear)
													61	2	30 (remainder)
Patients	399	3	133***	447	3	149***	184	3	61	276	3	92***	376	3	125
Interactions	108	6	18	64	6	11							263	6	44
Overlap x no.of injections							84	1	84	0	1	0			
Overlap x dose of HCG (1)							527	1	527**	32	1	32			
Overlap x dose of HCG (2)							40	1	40	6	1	6			
Overlap x dose of FSH							90	1	90	0	1	0			
No. of injections x dose of HCG							450	1	450**	39	1	39*			
Dose of FSH x dose of HCG							170	1	170*	3	1	3			
Residual	125	16	7.8	195	16	12.2	431	16	27	115	16	7	401	16	25
Total	688	31		842	31		3,830	31		705	31		1,378	31	

FIG. 15A

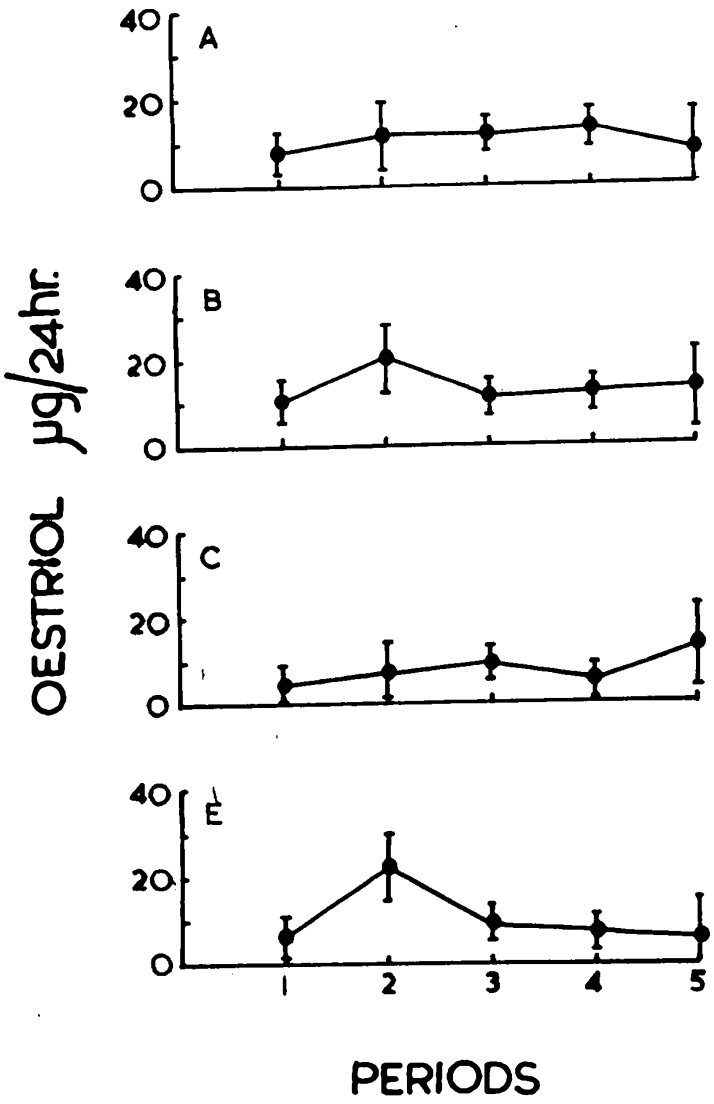


FIG. 15B

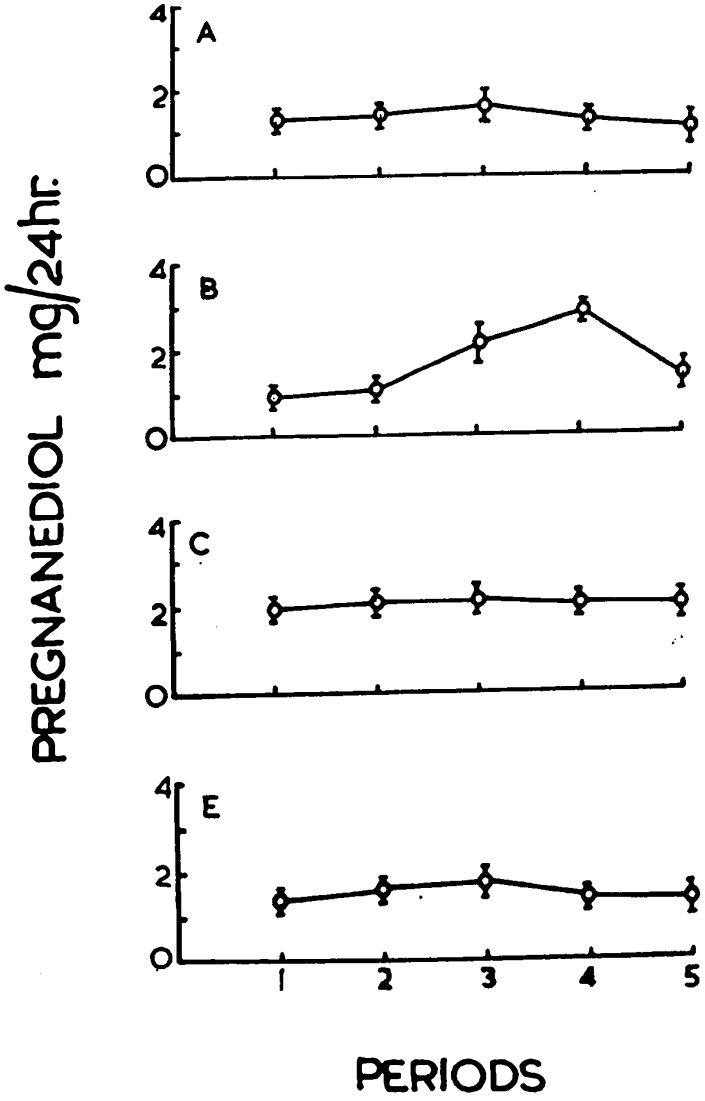


Fig. 15A.

Average excretion of oestriol in periods 1 to 5 in the four patients.

Fig. 15B.

Average excretion of pregnanediol in periods 1 to 5 in the four patients.

FIG. 16A

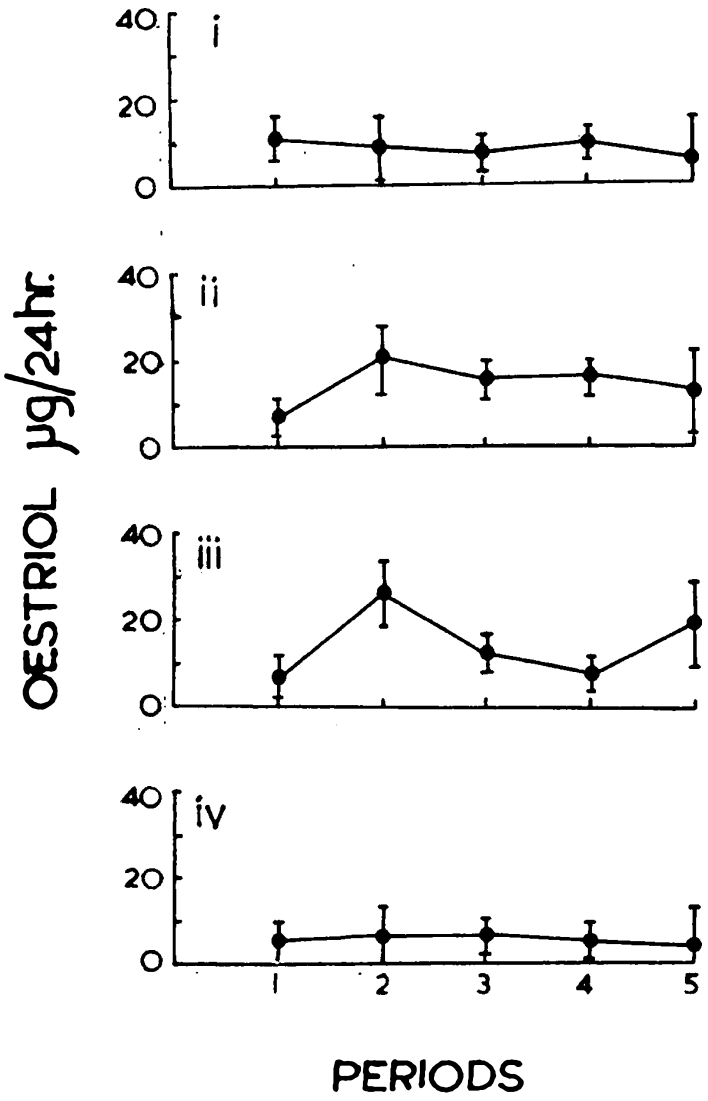
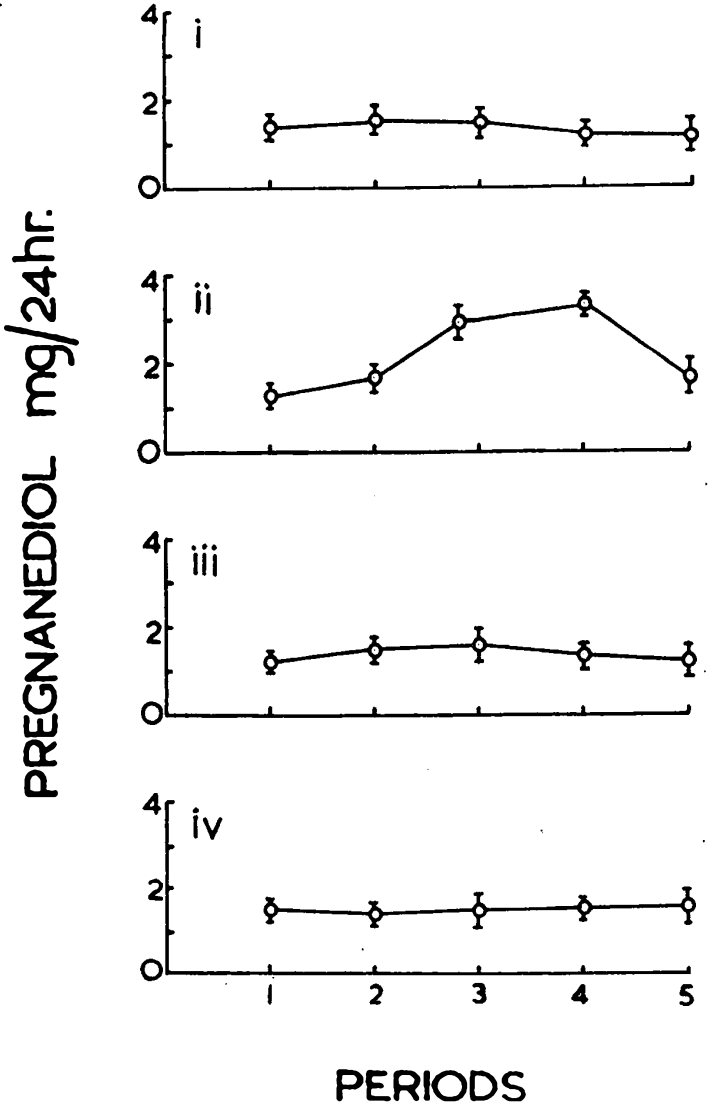


FIG. 16B



Effect of dosage and timing of treatment with HCG on the average excretion of oestriol and pregnanediol in the four patients.

(i) HCG (average dosage 4,500 i.u.) given partly or wholly after FSH.

(ii) HCG (average dosage 18,000 i.u.) given partly or wholly after FSH.

(iii) HCG (average dosage 4,500 i.u.) given simultaneously with FSH.

(iv) HCG (average dosage 18,000 i.u.) given simultaneously with FSH.

Period 5. The effect of an increased dosage of H.C.G. on the average excretion on oestriol depended only on the timing. As in Periods 2 and 3 the higher dosages given partly or wholly after F.S.H. gave a higher average excretion then when given simultaneously with it, while the lower dosages given simultaneously with F.S.H. gave higher averages than when given partly or wholly after it (Figure 16A).

There was no evidence that any of the other factors affected the excretion of oestriol during this period.

Pregnanediol:

Period 1: Patient C had a higher average excretion than the other three patients (Figure 15B).

Period 2: Patient C again had a higher average excretion than the other three.

It is possible that the higher dosages of F.S.H. produced a higher average excretion of pregnanediol (Figure 18B).

Period 3: The effect of increased dosages of H.C.G. was dependent on the same factors which operated for oestriol in the same period. The higher dosages (average 18,000 i.u.) given partly or wholly after F.S.H. (Figure 16B, ii) and in a single injection (Figure 17B, ii) produced higher average responses than the other alternatives. The higher dosages of H.C.G. (average 15,000 i.u.) combined with the high dosages of F.S.H. produced the highest average excretion (Figure 18B, iv).

Period 4: The pattern of results was dominated by the exceptionally high response of patient B. Apart from this patient C still maintained a higher average excretion than the others. Even if the response of patient B was discounted it is clear that the excretion was highest with the high dosages of F.S.H. and the highest dosages of H.C.G. (15,000 i.u.), (Figure 18B,iv).

Futhermore, the higher dosage of H.C.G. (average 15,000 i.u.) given in a single injection produced higher average figures than the other alternatives (Figure 17B,ii).

Period 5: Patient C still had a higher excretion than the other three (Figure 15B) but the average excretion increased with the dosage of H.C.G. and was approximately proportional to the logarithm of the dose (Table V11).

FIG. 17A

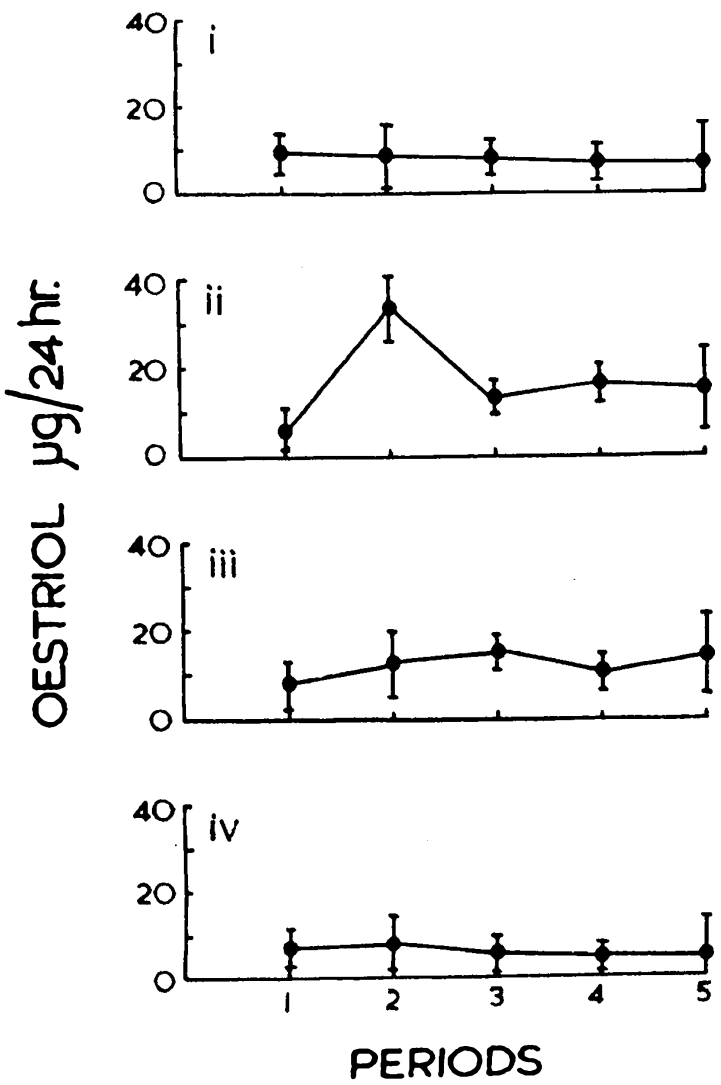
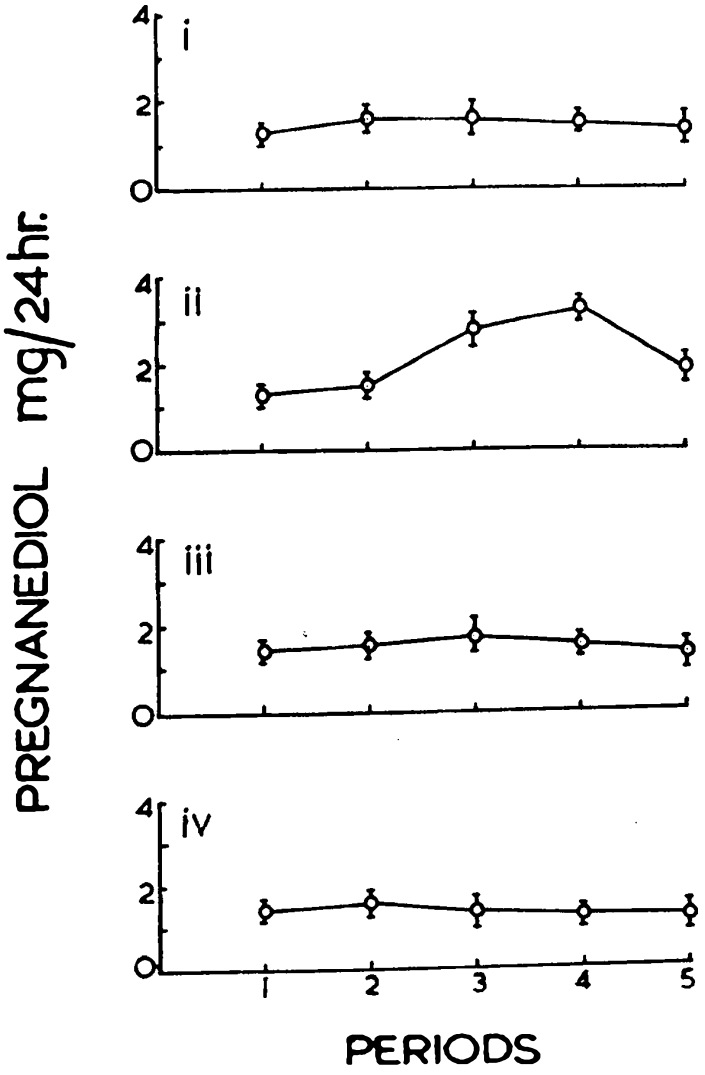


FIG. 17B



Effect of dosage and number of injections of HCG on the average excretion of oestriol and pregnanediol in the four patients.

(i) HCG (average dosage 7,500 i.u.) given as a single injection.

(ii) HCG (average dosage 15,000 i.u.) given as a single injection.

(iii) HCG (average dosage 7,500 i.u.) given in four injections.

(iv) HCG (average dosage 15,000 i.u.) given in four injections.

FIG. 18A

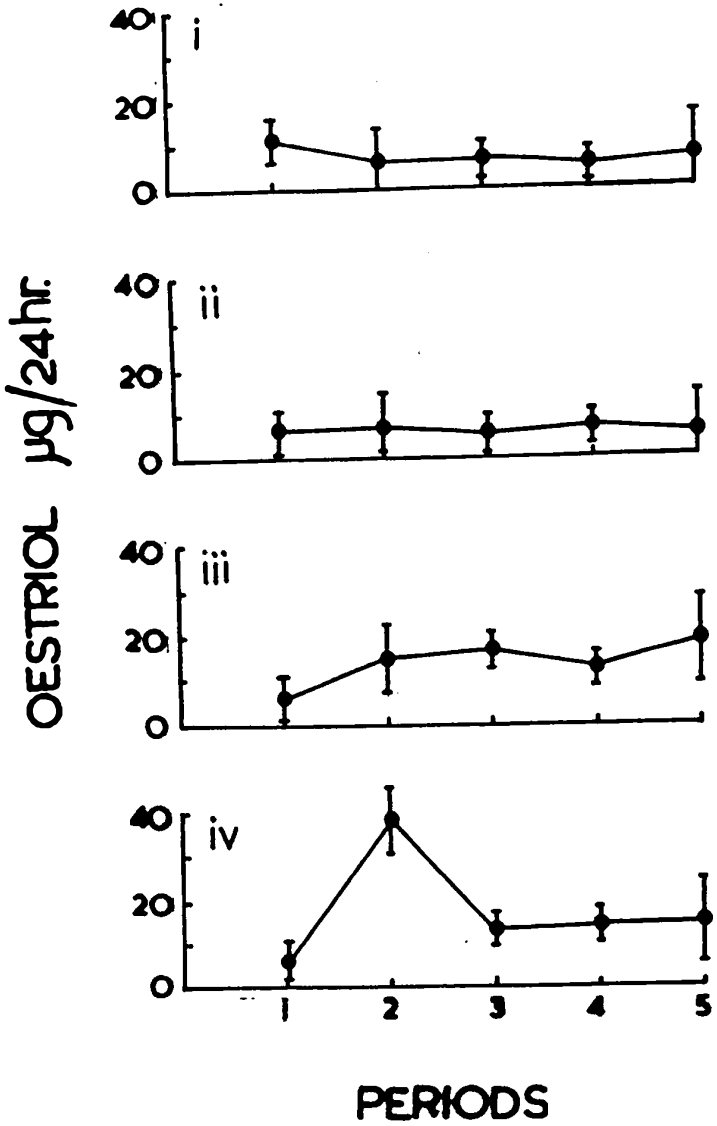
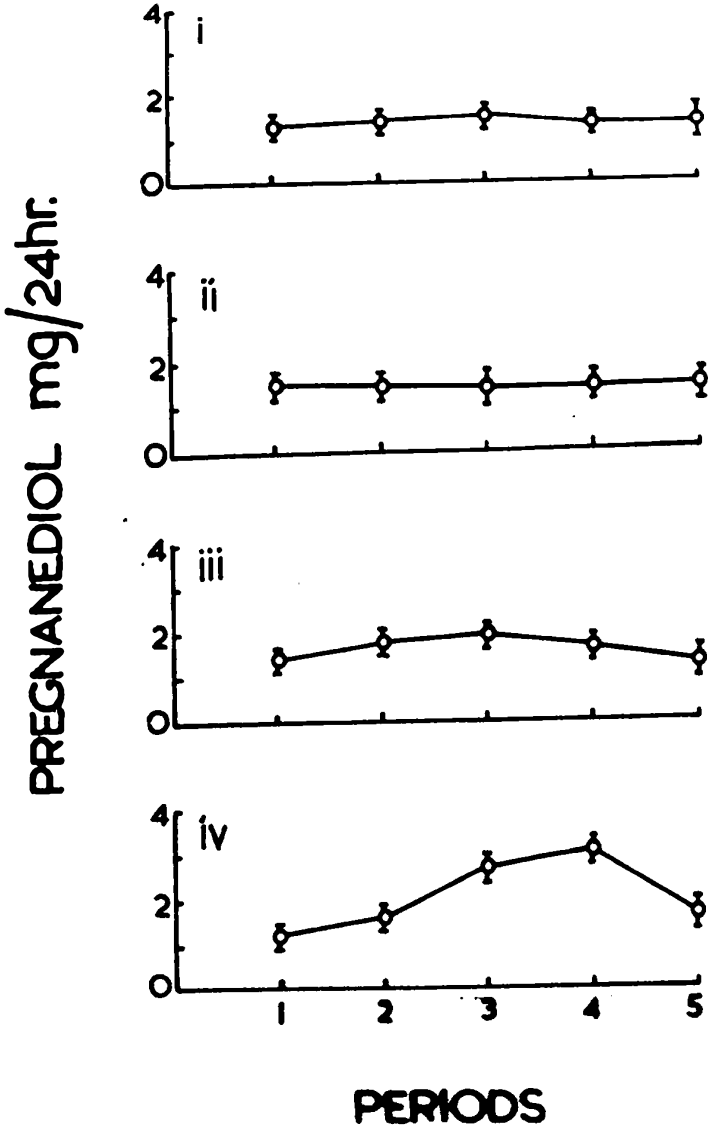


FIG. 18B



Effect of dosage of HCG and of FSH on average excretion of oestriol and pregnanediol in the four patients.

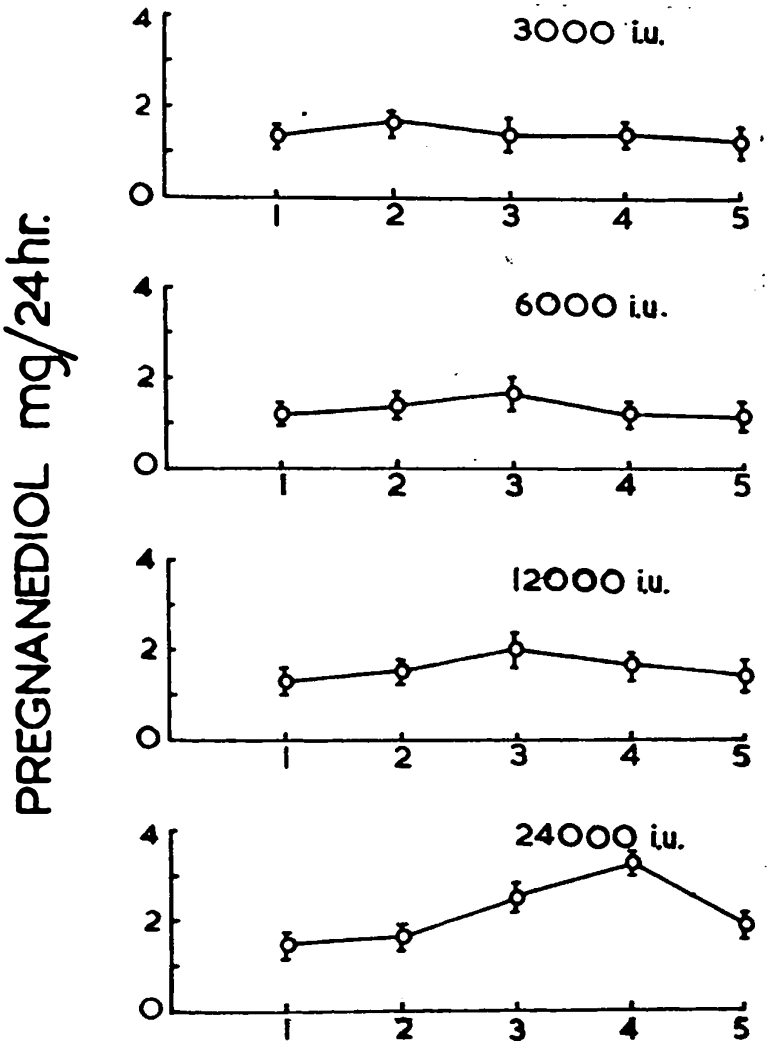
(i) HCG (average dosage 7,500 i.u.) and low dosage of FSH.

(ii) HCG (average dosage 15,000 i.u.) and low dosage of FSH.

(iii) HCG (average dosage 7,500 i.u.) and high dosage of FSH.

(iv) HCG (average dosage 15,000 i.u.) and high dosage of FSH.

FIG. 19



Effect of total dosage of chorionic gonadotrophin on the average excretion of pregnanediol in the four patients

Conclusion.

The results have been presented graphically in Figures 15 - 18 to facilitate comparison with the normal pattern of excretion of oestriol and pregnanediol. In addition Figure 19 shows the effect of the dosage level of H.C.G. on pregnanediol.

From these diagrams, the following appear to be the most promising treatments :-

1. H.C.G. in a dosage of 18,000 units, given partly or wholly after F.S.H. (Figure 15, ii).
2. H.C.G. in a dosage of 15,000 units, given as a single injection (Figure 17, ii).
3. H.C.G. in a dosage of 15,000 units, given with the higher level of F.S.H. (Figure 18, iv).
4. H.C.G. in a dosage of 24,000 units (Figure 19).

Combining these deductions the best treatment within the scope of the experiment would be to give :-

H.C.G. in a dosage of 18,000 units (or higher) in a single injection, after F.S.H. and using the higher level of F.S.H.

PREGNANCIES RESULTING FROM TREATMENT WITH GONADOTROPHIN

Treatment with gonadotrophin was continued in the three patients of Experiments 1 - 3 using the information gained from these experiments. Patients A, B and C subsequently became pregnant and the excretion of steroids and H.C.G. was followed throughout pregnancy.

The dates for the occurrence of each pregnancy have been calculated from a hypothetical last menstruation cycle (LMP) based on the finding in Experiments 1,2,3 that menstrual bleeding frequently occurred on the 24th day of the induced cycle. In the graphs week 0 corresponds to 5 days before the start of hormone treatment. The upper and lower 95% fiducial limits and the mean excretion of oestriol and pregnanediol in normal pregnancies are indicated (Coyle and Brown, 1963; Coyle, Mitchell, Russell, 1956). The dashed line for oestriol shows the lower limit of normal excretion where small babies were delivered. Each pregnancy will be described in the order in which it occurred.

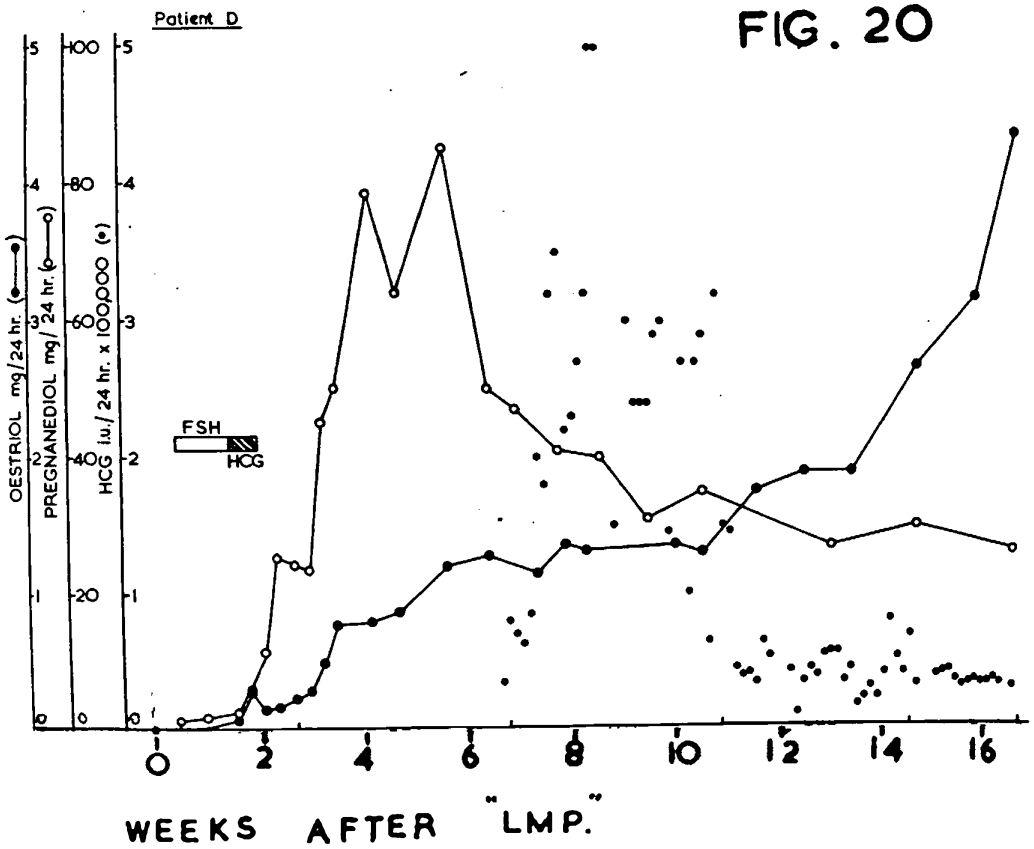
Pregnancy in Patient D:

During Experiment 2, month 3, patient D showed abnormally elevated levels of oestriol and pregnanediol (Figure D8, Expt. 2) and pregnancy was later confirmed. Oestriol and Pregnanediol were estimated twice every week in the first trimester of pregnancy and subsequently once a week.

The results during the first four months of pregnancy are shown in Figure 20. Pregnanediol rose to an abnormally high level reaching a maximum of 84.0mg/24 hr. between week 5 - 6, and fell to a level of 30mg/24 hr. in week 13. It remained at about this level throughout the latter period of pregnancy. A sample of blood taken from the patient during the ninth week was estimated to contain 10.2 µg. progesterone/100ml. This was between 2 - 3 times the value that is normally found in women at a corresponding stage of pregnancy. The excretion of oestriol, pregnanediol and H.C.G. are shown in Figure 21, a,b and c.

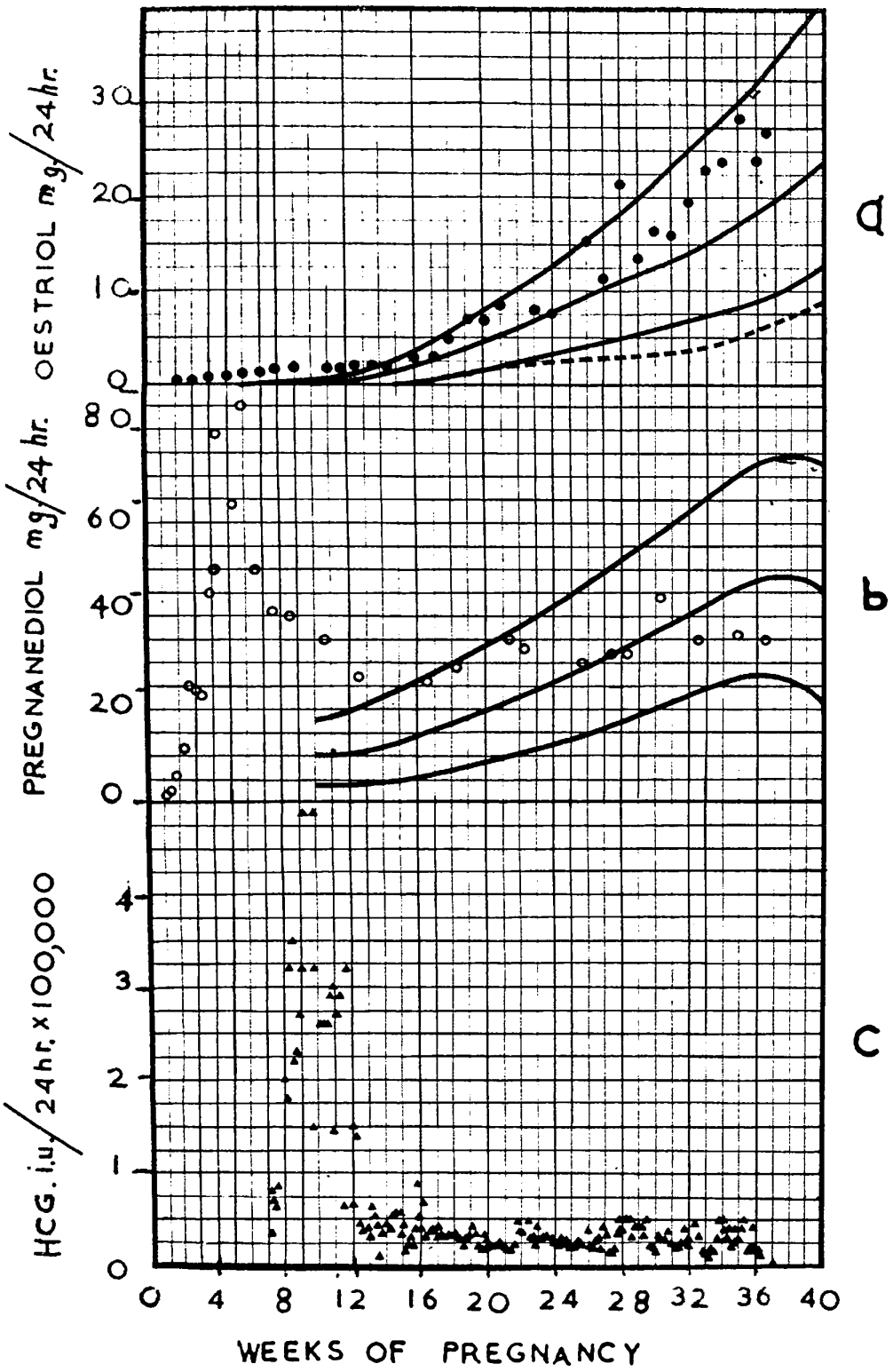
The excretion of H.C.G. reached a maximum of

FIG. 20



The excretion of oestriol, pregnanediol and HCG during early pregnancy in patient D.

PATIENT D FIG. 21



The excretion of (a) oestriol, (b) pregnanediol and (c) HCG during pregnancy. Also shown are the upper, mean, and lower limits for the excretion of oestriol and pregnanediol in a series of normal pregnancies, (see text). The broken line in (a) represents the lower limit for small babies. The limits are also shown in figs. 24 and 26.

500,000 i.u./24 hr. at about the eighth week and remained at a high level for about a further three weeks. From week 14 to the time of delivery the level of H.C.G. fluctuated between 20,000 and 50,000 i.u./ 24 hr. (Figure 21C). At the 9th week oestriol was above the upper limit of normal but by the 14th week it had approached the normal mean (Figure 21 a). For the remainder of the pregnancy the level increased steadily between the normal mean and the upper limit of normal. The excretion of pregnanediol at the 17th week had fallen to within the upper limits of normal and stayed fairly constant at about 34mg/24 hr. for the remainder of the pregnancy (Figure 21 b).

This patient gave birth to dissimilar twins at 37 weeks.

Pregnancy of Patient C.

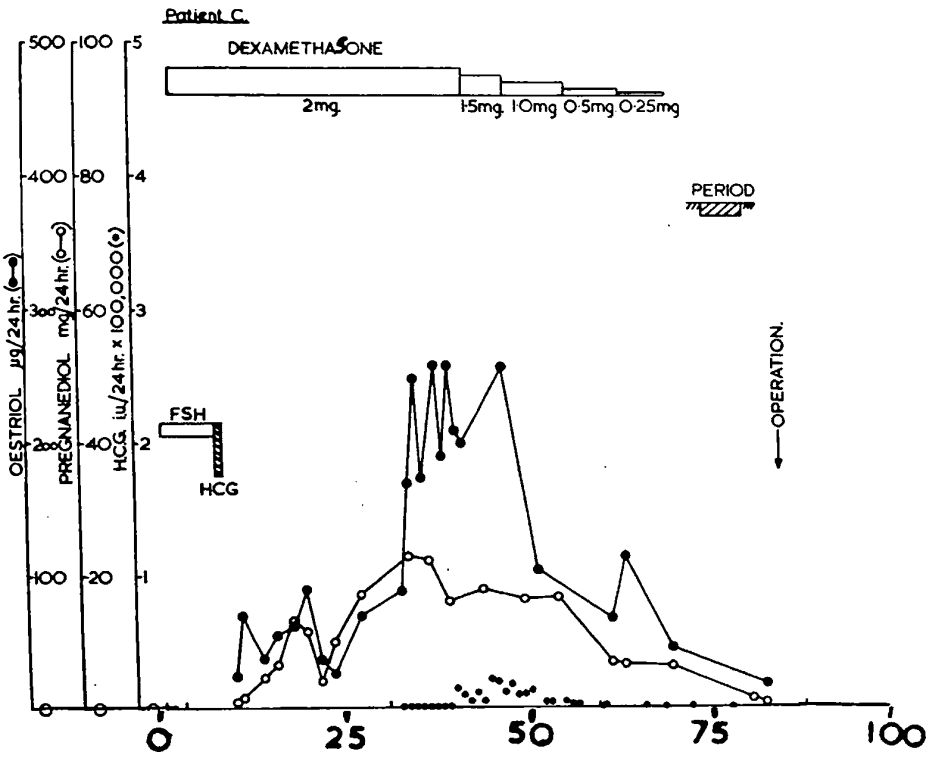
Treatment:

This patient was treated with F.S.H. (800mg. equivalents 1 RP-HMG/day) for 8 days and with H.C.G. (12,000 i.u./day) on day 9. A daily dose of 2.0mg. dexamethasone was also given to this patient until the 44th day and it was then gradually reduced, first to 1.5mg/day on day 44, then to 1.0mg/day on day 50, 0.5mg/day on day 59, and 0.25mg/day on day 68. Treatment was finally stopped on day 74.

The excretion of oestriol, pregnanediol and H.C.G. is shown in Figure 22. Dexamethasone reduced the control level of pregnanediol to 0.9mg/24 hr. After treatment with gonadotrophin oestriol rose to 70.0µg/24 hr. in the ovulatory phase of the induced cycle and pregnanediol reached 13.2mg/24 hr. during the luteal phase. The levels of both steroids were elevated on day 28 and estimations were continued until day 84.

After day 28 the steroid levels increased steadily, oestriol reached a maximum of 242µg/24 hr. on day 37 and pregnanediol a maximum of 20mg/24 hr. Both levels fell together and had returned to pretreatment levels at the time of operation. H.C.G. was barely detectable on most days but estimates were obtained by concentrating the gonadotrophin using alcohol precipitation. The level of H.C.G. fluctuated between 10,000 - 20,000 i.u./day between days 44 - 66. Uterine bleeding commenced on the 70th day and the patient aborted. Surgical examination on the 84th day revealed portions of decidua, hyalanised chorionic villi gestation sac and fragments of trophoblast.

FIG. 22



The excretion of oestriol, pregnanediol and HCG in patient C.
The treatment with dexamethasone is also shown.

Pregnancy of Patient A.

The results of the previous trials on this patient suggested that high dosages of F.S.H. were required to cause ovulation. These were increased accordingly to 1,000mg. equivalents 1RP-HMG given on day 1,3,5 and 7 followed by H.C.G. 12,000 i.u. on day 8.

The excretion of oestriol, pregnanediol and H.C.G. are shown during early pregnancy (Figure 23) and throughout the entire pregnancy in (Figure 24 a,b and c).

The excretion of H.C.G. increased from week 6 and reached a peak value of 370,000 i.u./24 hr. between weeks 8-9. By the 11th week it had fallen to about 20,000 i.u./24 hr. at which level it remained, apart from a slight increase noted during week 34.

Pregnanediol excretion was between the mean and the lower limits of normal until the 31st week when it fell rapidly to 15.2mg/24 hr. Over the same period oestriol, which had been between the mean and the upper limits of normal, fell from 14.9mg/24 hr. to 6.5mg/24 hr. These changes were more than could be accounted for by daily fluctuation in steroid output during pregnancy so the patient was admitted to hospital and steroid therapy was commenced. The treatment is shown in Table:-

Table VIA

Steroid Therapy

Expressed as mg./week.

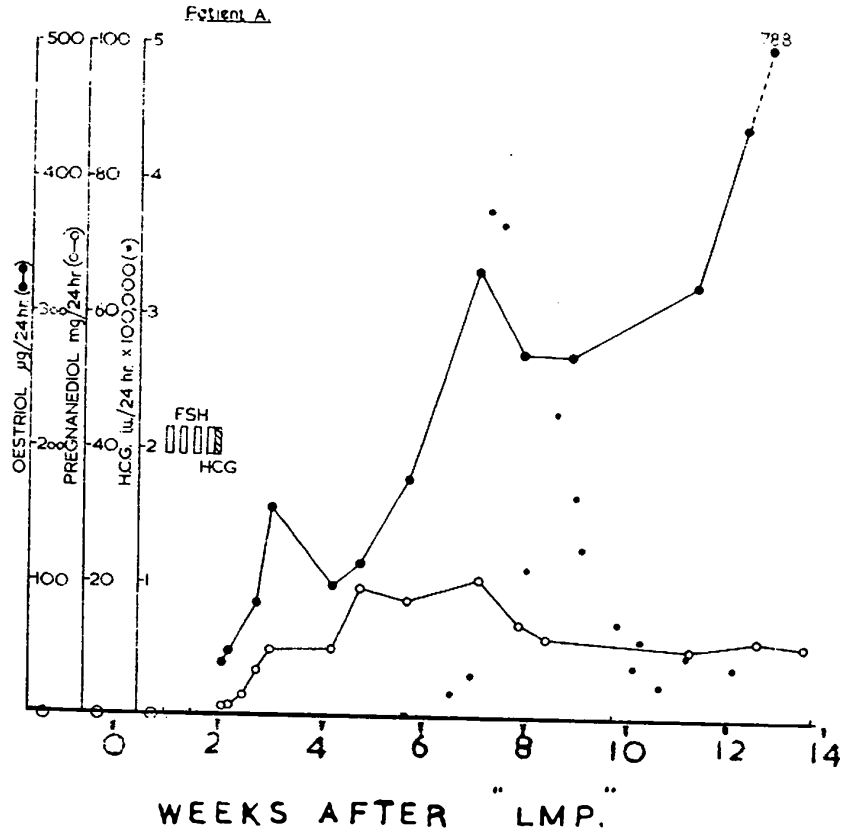
Patient A.

Treatment.	Week 32-33	Week 33-34	Week 34-35	Week 35-36	Week 36-Delivery
Ethinyl Oestradiol		12	34	26	1
Oestradiol Monobenzoate	10	20	20	15	
Norethisterone		200	280	520	20
17 Hydroxy Progesterone Caproate			500	375	
Progesterone	20	50			

This table shows the quantities (expressed in mg./week) of different steroids administered to Patient A.

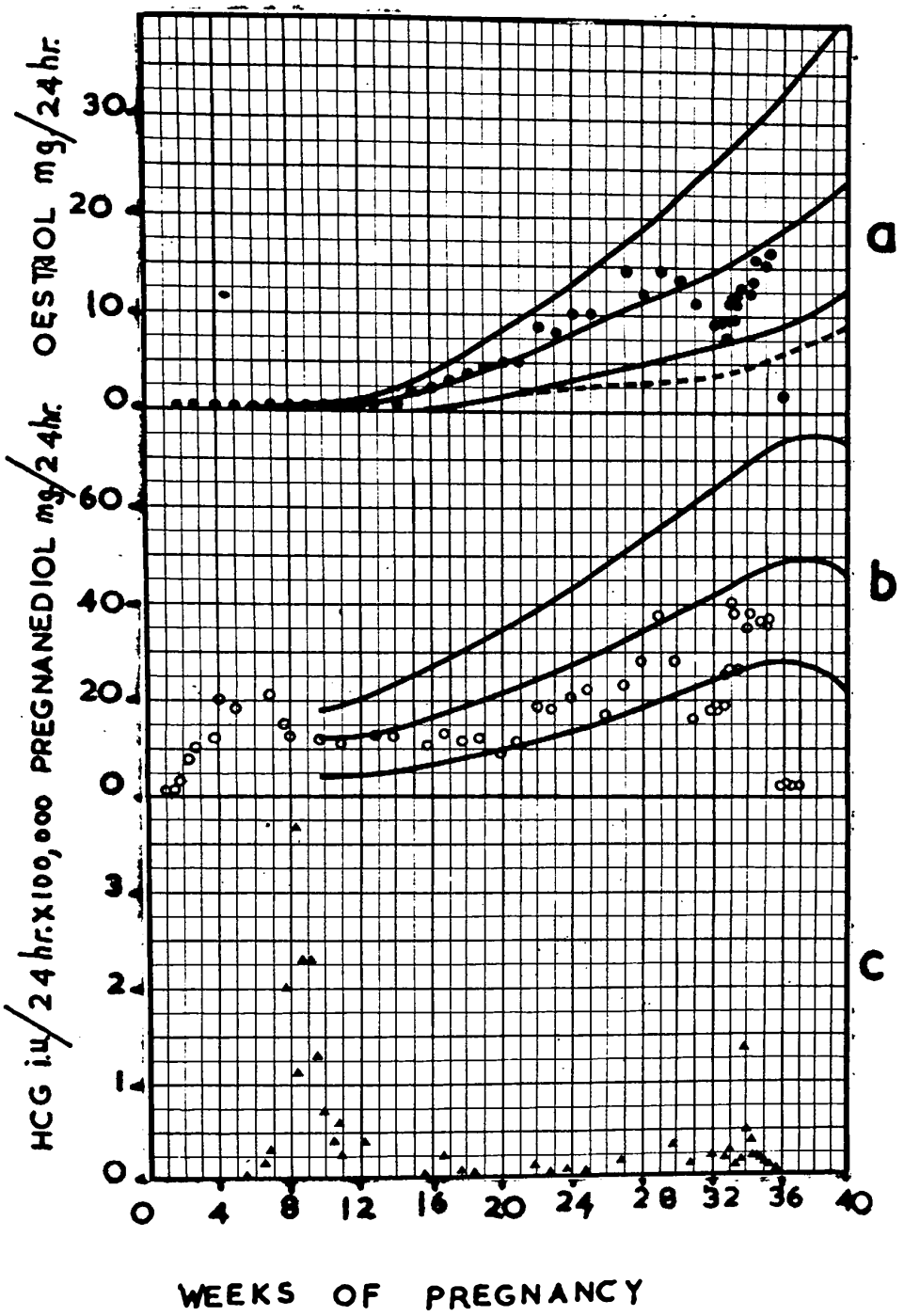
The excretion of both steroids returned to normal levels during treatment and a female baby was delivered by Caesarean section at 36 weeks.

FIG. 23



The excretion of oestriol, pregnanediol and HCG during early pregnancy in patient A.

PATIENT A FIG. 24.



Excretion of (a) oestriol, (b) pregnanediol and (c) HCG during pregnancy in patient A.

Pregnancy of Patient B.

This patient was treated with F.S.H. 800mg. equivalents 1RP-HMG/day for 8 days followed by 24,000 i.u. H.C.G. on day 9. The excretion of oestriol, pregnanediol and H.C.G. is shown in Figures 25 and 26 a,b and c.

The excretion of oestriol and pregnanediol increased until the 6th week but then both began to fall. Uterine bleeding occurred at this time and the patient was confined to bed. H.C.G. was first detected at about the 7th week and reached a peak of 200,000 i.u./24 hr. at the 10th week. It fell to between 10-20,000 i.u./ 24 hr. about the 14th week and apart from some high values in the 16th week remained at this lower level until the 30th week when it increased again during steroid therapy. The steroids remained at the lower limits of normal until the 28th week when they fell. At this stage the patient was admitted to hospital and steroid therapy was begun at 31 weeks. The treatment is shown in Table:

Table VIIA

Steroid Therapy

Expressed as mg./week.

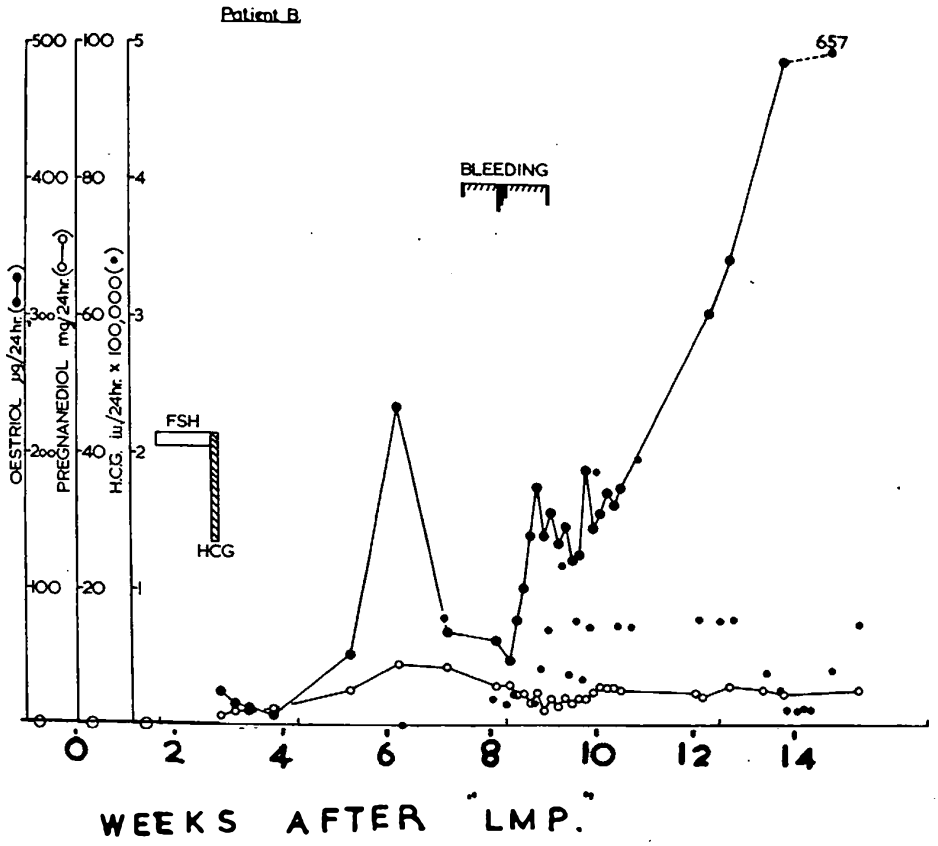
Patient B.

Treatment.	Week 31-32	Week 32-33	Week 33-34	Week 34-Delivery.
Oestradiol Undecylenate	100			
Ethinyl-oestradiol	12	8	84	
Oestradiol Monobenzoate	20	20	40	20
17 Hydroxy Progesterone Carpoate	1000	1000	1550	1000

This table shows the quantities (expressed as mg./week) of different steroids administered to Patient B.

During treatment with steroids the pregnanediol level increased slightly but little change occurred in the level of oestriol. At 34½ weeks a male child was delivered by Caesarean section.

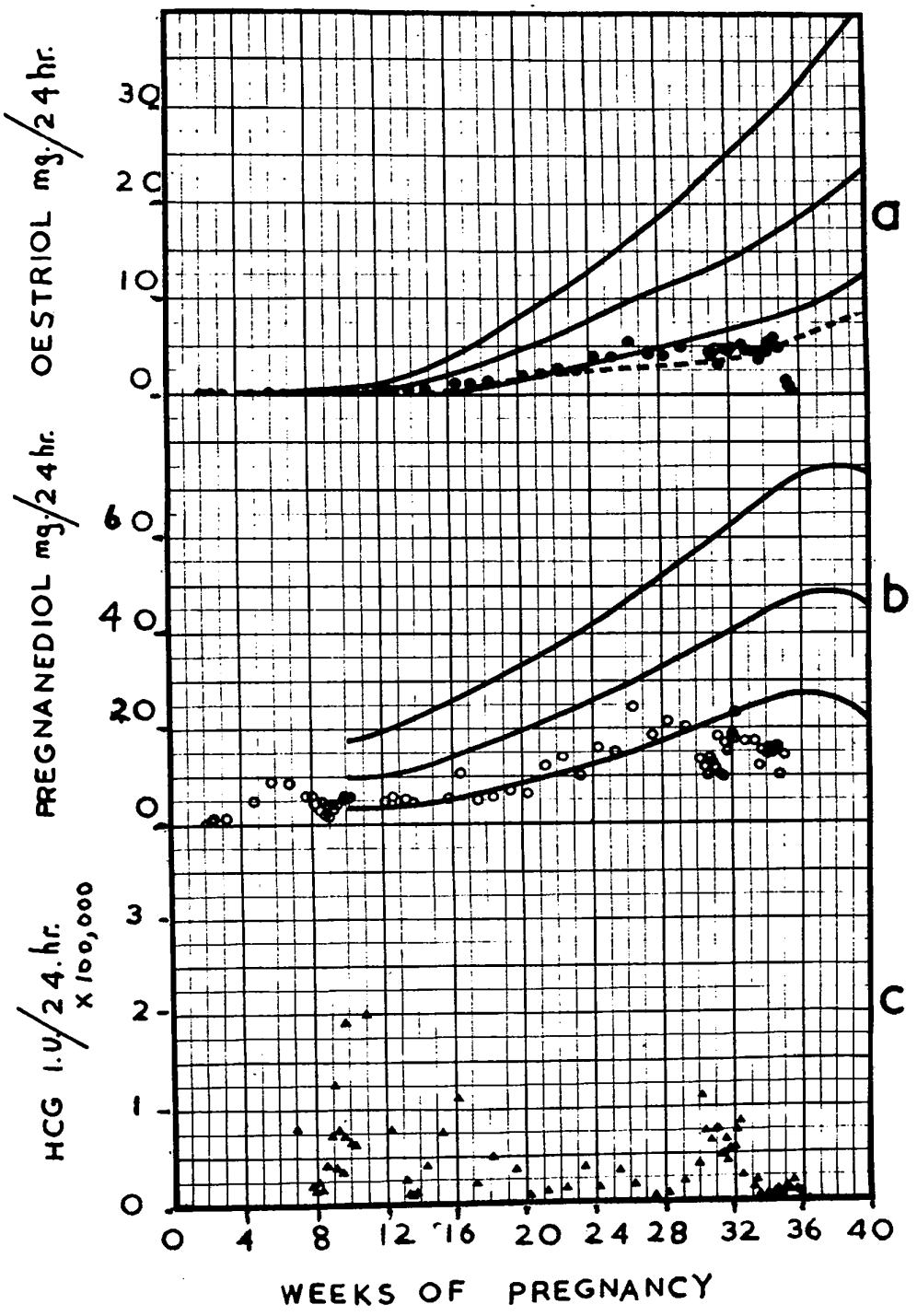
FIG. 25



The excretion of oestriol, pregnanediol and HCG during early pregnancy in patient B.
The duration of uterine bleeding is also shown.

PATIENT B

FIG. 26.



The excretion of (a) oestriol (b) pregnanediol and (c) HCG during pregnancy in patient B.

RESULTS

SECTION III

SECTION III

The effect of treatment with gonadotrophin on the production of ovarian steroids by a woman with Simmonds Disease.

The Patient.

This patient was aged 27 and had had a chromophobe adenoma of her pituitary gland removed six years previously. She had no detectable gonadotrophins and the excretion of 17-oxosteroids and 17-oxogenic steroids was below normal. The radio-iodine uptake of the thyroid gland was at the lower limit of the normal range. She was given cortisone and thyroxin at various times but discontinued treatment voluntarily.

The patient (F) was treated with F.S.H. 600mg. equivalents/RP-HMG/day for 12 days followed by H.C.G. 6,000 i.u./day for 3 days. Urine was collected every day and oestriol and pregnanediol was estimated on each specimen. The treatment and results are shown in Figure 27.

The excretion of oestriol was initially 2.3µg./24hr. and after treatment for 7 days with F.S.H. (day 14) it rose to 25µg./24hr. After treatment with H.C.G. it rose to a maximum of 38µg./24hr. on day 26. Pregnanediol on the other hand showed no change until after treatment with H.C.G. and reached a maximum value of 1.4mg./24hr. on days 23 and 26. It is doubtful if this slight rise on pregnanediol represented an ovulatory change and further experiments were therefore undertaken.

The period of treatment with F.S.H. was 12 days in the first month because certain metabolic studies were in progress concurrently. Since however treatment over 8 days had been successful in the clinical trials reported in the previous section it was decided to reduce the length of treatment to 8 days in subsequent experiments. Single specimens of urine were collected on days 5, 18, 20, 22, 24 and 28 and the specimens of urine collected on days 9 and 10, 11 and 12, and 15 and 16 were pooled. Results were always expressed as the amount of steroid excreted for 24hr. Day 28 was taken as the control day for the following month of treatment.

FIG. 27

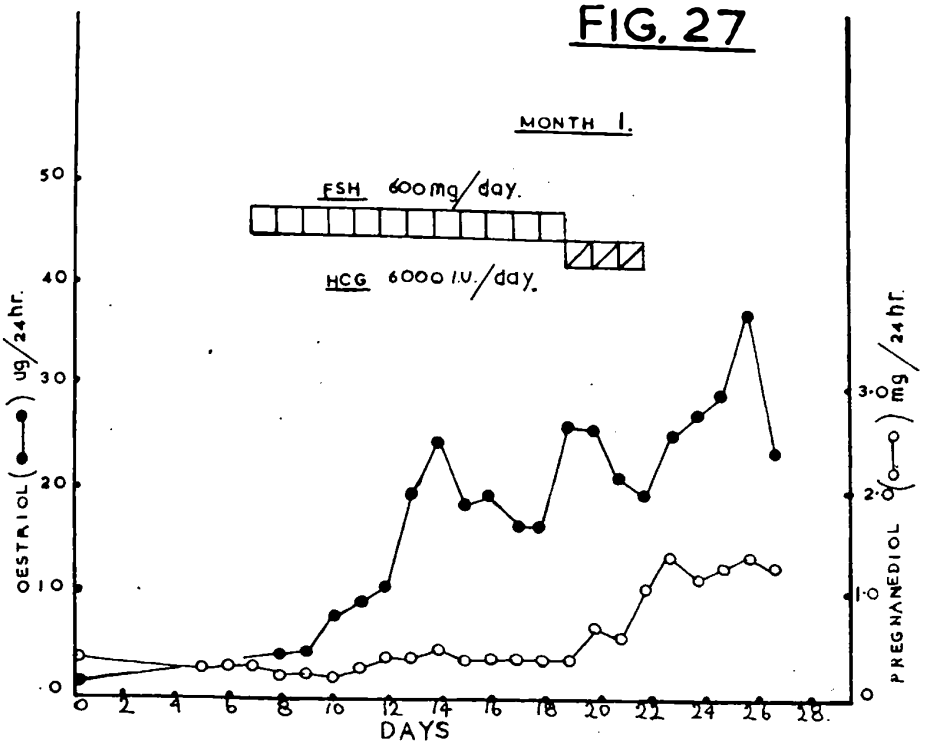


FIG. 28

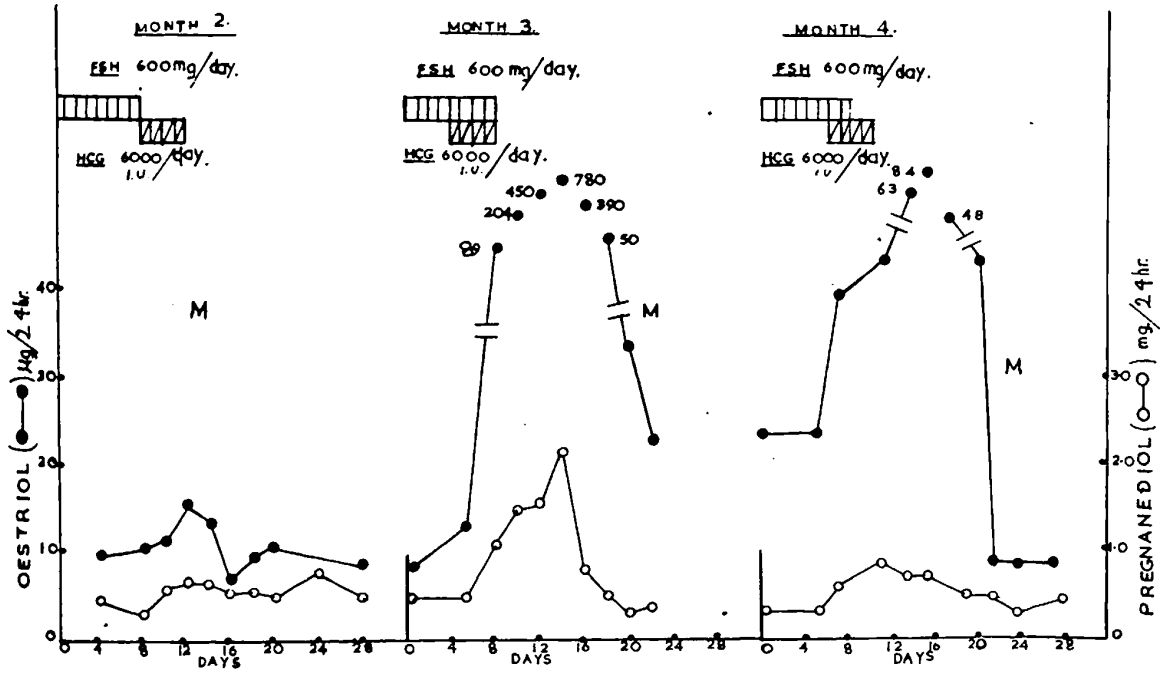


Fig. 27. The effect of FSH and HCG on the excretion of oestriol and pregnanediol in patient F.

Fig. 28. The excretion of oestriol and pregnanediol after treatment with FSH and HCG.
Month 2: HCG was given after FSH
Month 3: HCG was given simultaneously with FSH
Month 4: HCG was given partly with and partly after FSH.

Months 2 - 4.

These trials were to investigate the effect of altering the time of treatment with H.C.G. relative to F.S.H.

F.S.H. was given at a dosage of 600mg. equivalent 1RP-HMG for 8 days and H.C.G. 6,000 i.u./day for 4 days. The H.C.G. was given in different ways as follows:-

Month 2: No overlap: H.C.G. was given immediately after treatment with F.S.H.

Month 3: Complete overlap: H.C.G. was given with the last 4 injections of F.S.H

Month 4: Partial overlap: H.C.G. was given with the last two injections of F.S.H. and and for 2 days after F.S.H.

The results, treatment and doses of hormones used are shown in Figure 28.

Month 2: No overlap:

The response was poor since oestriol increased to only 16µg./24hr during treatment with H.C.G. while little change occurred in the level of pregnanediol. Menstruation (M) occurred on the day after treatment with H.C.G.

Month 3: Complete overlap:

In contrast to month 2 an enormous increase in the excretion of oestriol during this trial. On day 14 a maximum excretion of 780µg./24hr. was reached and the level remained above the control level for fourteen days. There was no change in the excretion of pregnanediol during treatment with F.S.H. alone but it increased steadily after treatment with H.C.G. and reached a peak value of 2.2mg./24hr. on day 14. Menstruation occurred on day 23.

Month 4: Partial overlap:

Treatment this month was commenced while the excretion of oestriol was still elevated following the previous treatment. After treatment with H.C.G. it rose rapidly and reached a maximum of 84µg./24hr. on day 16. Only a slight increase in pregnanediol occurred during treatment with H.C.G. and it decreased again

immediately after treatment. Menstruation occurred on day 24.

It is evident that treatment with gonadotrophin was followed by an enormous response in the excretion of oestriol but there was little change in pregnanediol. The pattern of response therefore is not typical of that following normal ovulation.

Further trials were planned to investigate whether the failure of ovulation was due to the deficiency of some other pituitary hormone. Treatment with F.S.H. and H.C.G. was according to the schedule that was most effective in producing a rise in the excretion of oestriol and pregnanediol i.e. complete overlap of the two hormones.

The results are shown in Figure 29.

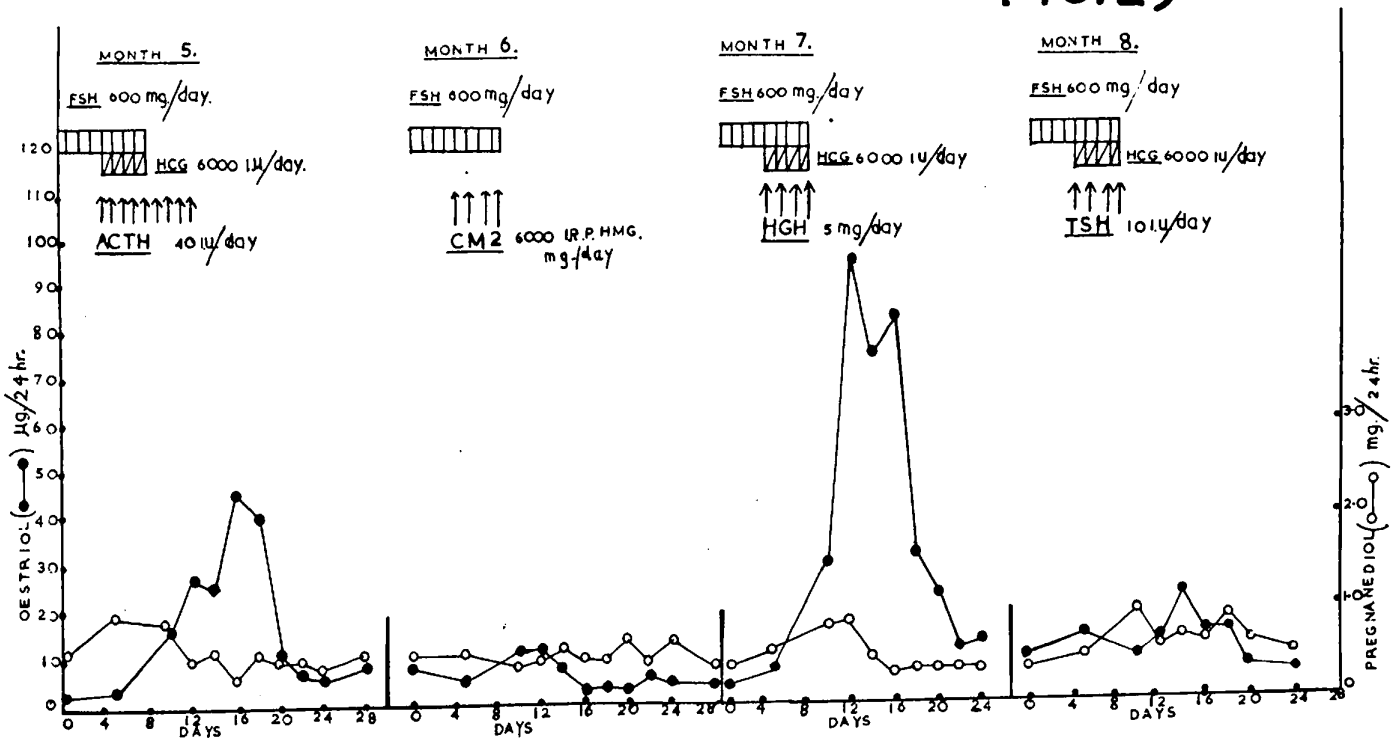
Month 5: A.C.T.H. (40i.u./day) was given for 8 days commencing on the first day of treatment with H.C.G. The excretion of oestriol increased steadily after treatment with gonadotrophin and reached a maximum of 46µg./24hr. on day 16. Again pregnanediol failed to rise after the increase in oestriol.

Month 6: A preparation of human pituitary LH (CM2 fraction) was used instead of H.C.G., the daily dosage was equivalent to 6,000 mg. equivalents 1RP-HMG. There was little change in the excretion of oestriol and pregnanediol either during treatment with F.S.H. alone or after the combined treatment with F.S.H. and pituitary L.H.

Month 7: In this month human growth hormone (H.G.H. 5mg./day) was given for four days on the same days as H.C.G. were given. The level of oestriol increased after the combined treatment with F.S.H., H.C.G., and H.G.H. and it reached a maximum value of 96.0µg./24hr. on day 12. There was a slight increase in pregnanediol on day 12 but on day 16 it had returned to the control level.

Month 8: Thyroid stimulating hormone (T.S.H. 10i.u/ day for 4 days) was given with H.C.G. A slight increase in the excretion of oestriol occurred on day 14 and in pregnanediol on day 10. These changes were not very pronounced.

FIG. 29



The effect of different hormone preparations on the excretion of oestriol and pregnanediol.

Conclusion:

With the doses of trophic hormones used the best oestriol response occurred in month 7 after treatment with H.G.H. and gonadotrophin and little change occurred after either T.S.H. or CM2. The addition of A.C.T.H. was followed by a normal oestriol response. The level of pregnanediol showed little change in any of the months of treatment.

In a patient with secondary amenorrhoea (Patient C Section II) it was found that ovulation and pregnancy occurred after the simultaneous administration of dexamethasone and gonadotrophin. This was, therefore, tried in this patient. Dexamethasone was used together with the gonadotrophin in months 9, 10 and 11. Treatment and results are shown in Figure 30.

Month 9: Treatment was similar to month 3 but 0.5mg. dexamethasone and 0.05µg. thyroxin was given daily during treatment.

The response in oestriol is nearly identical to the type of response attained in month 3 but in this month the excretion of pregnanediol reached a peak value of 3.0mg./24hr. but only remained elevated above control for 4 days. Menstruation occurred as the oestriol level dropped. The very sharp increase in both oestriol and pregnanediol were not of the type seen in normal ovulatory cycles.

In the next two months variations were tried in the method of administering F.S.H. and H.C.G. The clinical trials reported in the previous section had shown that F.S.H. could be effective when given on alternate days instead of every day and that H.C.G. could be given in one dose rather than four. While still on dexamethasone and thyroxin F.S.H. was now given in 4 injections of 1,200mg. equivalents 1RR-HMG and H.C.G. as 24,000 i.u. on the eighth day. This was followed by one month with F.S.H. in 8 injections of 600mg. equivalents 1RP-HMG and H.C.G. in one dose of 24,000 i.u. on the day following F.S.H.
The results are shown in Figure 30.

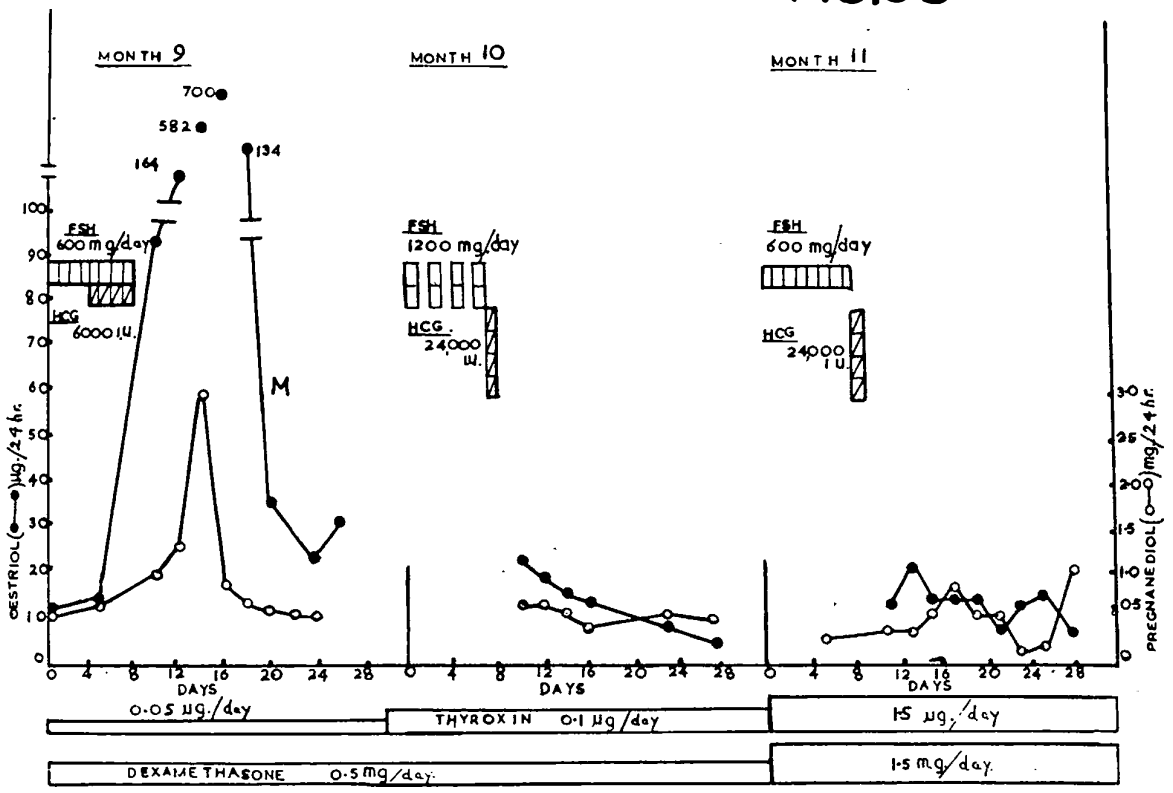
Month 10: There was no increase in either oestriol or pregnanediol following treatment but no estimations were performed between days 1 - 8 since the level of oestriol was probably still affected by the treatment of the previous month.

Month 11: There was a small increase in oestriol after treatment with H.C.G. and probably a small increase in pregnanediol following this.

Conclusion:

The best pattern of steroid response occurred when there was complete overlap of H.C.G. and F.S.H. and the patient was given dexamethasone and thyroxin. The

FIG.30



The excretion of oestriol and pregnanediol.
The different dosages of FSH, HCG, dexamethasone
and thyroxin used are also shown.

methods of giving F.S.H. on alternate days or F.S.H. every day and all of the H.C.G. in a single large dose did not appear to be effective in this patient. This was in marked contrast to the responses of the patients with secondary amenorrhoea.

Summary to Section III.

1. Treatment of a patient with Simmonds' disease with F.S.H. and H.C.G. was followed by changes in the excretion of oestriol and pregnanediol.
2. The best response was obtained when H.C.G. was given at the same time as F.S.H.
3. In the dosages used the additional trophic hormones caused no additional change in the production of steroids.
4. None of the treatments induced a pattern of excretion of steroids similar to that seen in a normal ovulatory cycle.

RESULTS

SECTION IV

The Patients:

Each of the patients in this section possessed the clinical symptoms of the Stein-Leventhal Syndrome.

The first three experiments in this section were designed to study the effects of treatment with F.S.H. alone and with combined F.S.H. and H.C.G. on the production of ovarian steroids. Little was known about the relative doses of F.S.H. to use or the way to combine F.S.H. and H.C.G. to achieve the best ovarian steroid production. Dexamethasone was incorporated into these experiments to suppress the production of adrenal steroids so that it may be assumed that the increased steroid levels found after treatment with gonadotrophin would be specifically of ovarian origin.

Doses of gonadotrophin used in Patients G, H and I.

F.S.H. was given in two injections, each equivalent to 2,000mg. 1RP-HMG, the second twelve days after the first. H.C.G. 12,000 i.u. was given in a single injection two days later. Treatment with dexamethasone (3mg./24hr.) was commenced at least 4 days before the first injection of F.S.H.

Collections of urine:

Urine was collected daily, beginning 2 days before treatment with dexamethasone. This served as a control and subsequent 24 hr. specimens of urine were pooled in 48 hr. lots but the results were expressed as excretion /24 hr. The days referred to in the graphs refer to the day of completion of the 48 hr. pool.

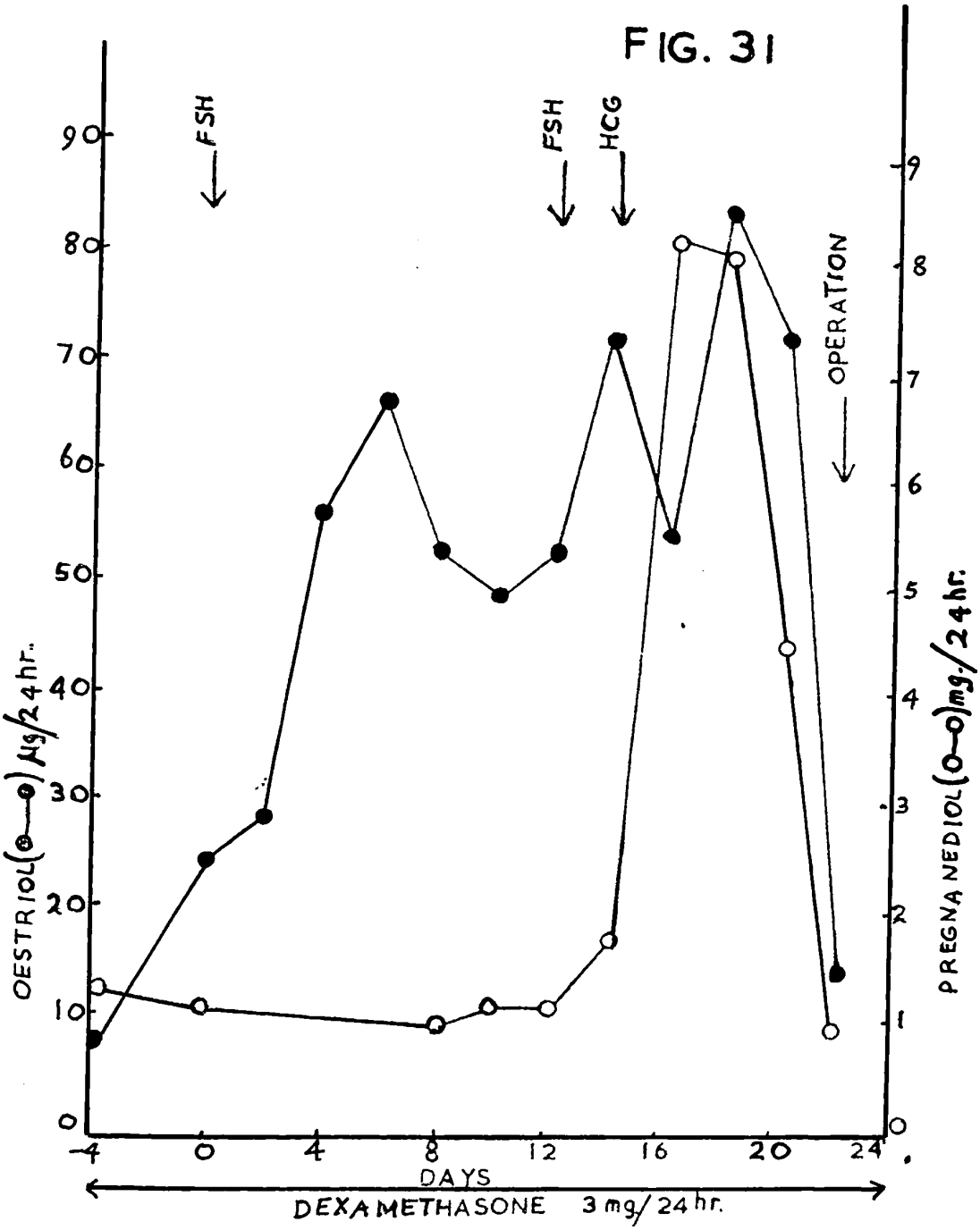
Case G:

The results and treatment are shown graphically (Figure 31): The excretion of oestriol and pregnanediol.

Before treatment commenced the excretion of pregnanediol was only 1.2mg./24hr. and oestriol was 8.0µg./24hr. After treatment with dexamethasone for four days the excretion of pregnanediol was not significantly changed but oestriol had increased threefold.

Following a single injection of F.S.H. no change occurred in the excretion of pregnanediol but that of oestriol level increased to 68.0µg./24 hr. on day 6. By day 8 it had fallen and on day 10 it was 50.0µg./24hr.

FIG. 31



The effect of FSH and HCG on the excretion of oestriol and pregnanediol in patient G. The dosage of dexamethasone used is also shown.

The injection of F.S.H. on day 12 brought about an increase in the level of oestriol to 73.0µg./24hr. and a slight increase in pregnanediol. After the injection of H.C.G. there was an immediate increase in pregnanediol to 8.2mg./24hr. on day 16 and an increase in oestriol to 85µg./24hr. The excretion of both steroids fell rapidly and by day 22 had almost returned to the control levels.

Laparotomy was performed on day 22 and the ovaries were found to be slightly enlarged and contained numerous small cysts and a single corpus luteum. There was haemorrhage into the lumen and some of the cysts and an area of haemorrhage in the ovarian stroma.

Ovarian cyst fluid was removed from this patient and was found to contain small quantities of progesterone, 17α-OH progesterone and androstenedione. Part of the luteal tissue removed was also found to contain progesterone. These results are summarized in Table VIII.

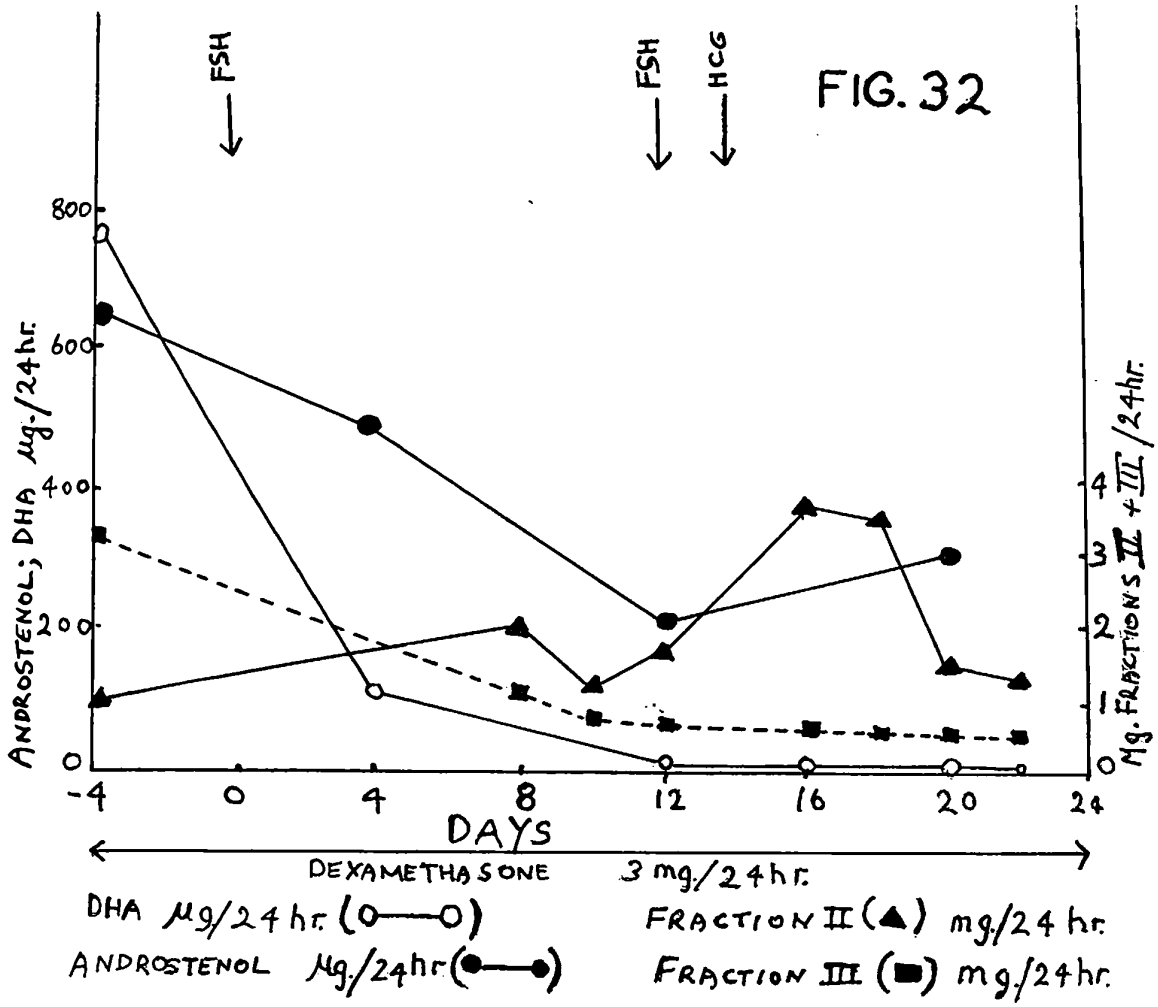
Figure 32 - The Excretion of Dehydroepiandrosterone, Androstenol, and Fractions II and Fractions III.

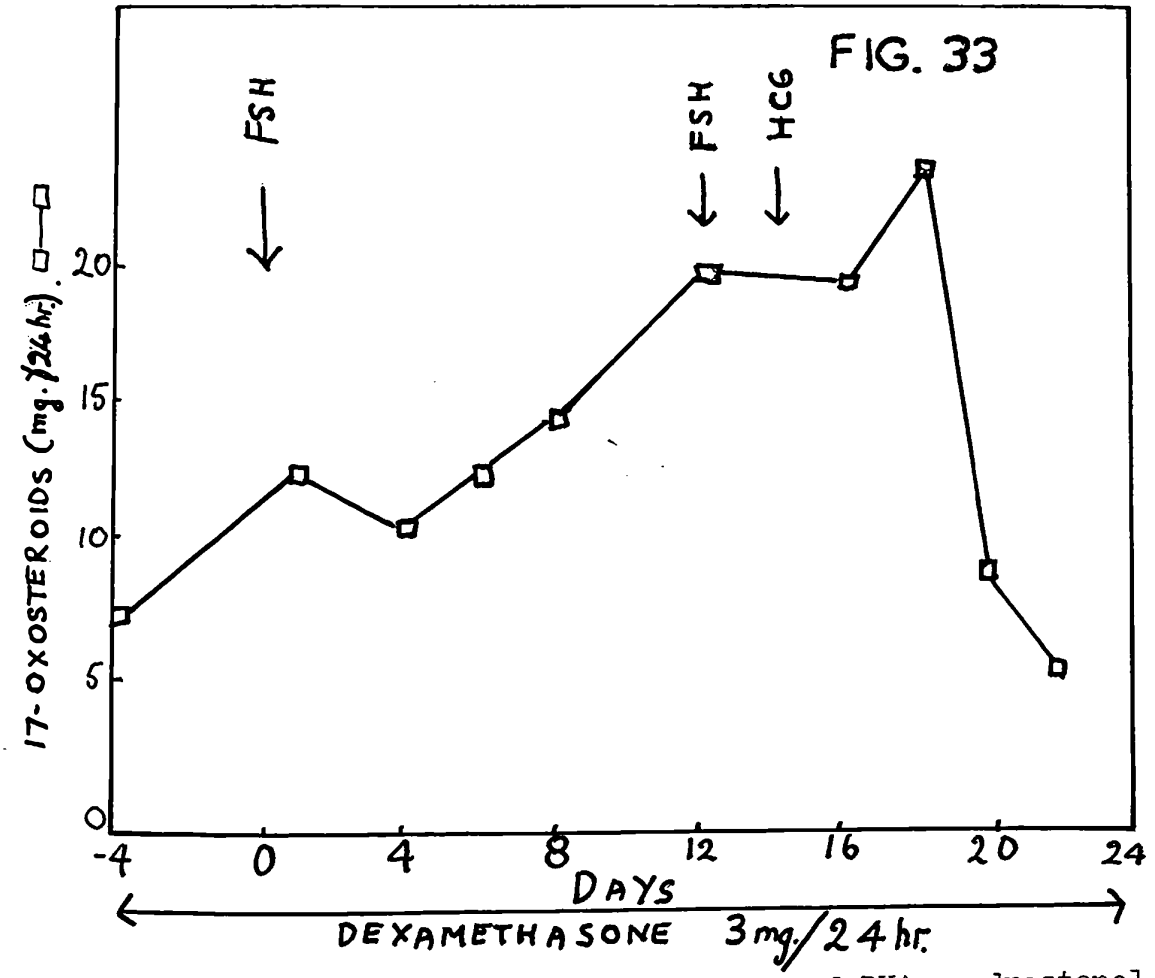
Dexamethasone suppressed the excretion of DHA completely and reduced that of androstenol from 630.µg./24hr. on day 12. Fraction III decreased steadily from 3.1 to 0.5mg. equivalents aetiocholonolone/24hr. on day 12 and remained at this level until the end of the period of treatment. The effect of the single injection of F.S.H. was not very clear as the steroid levels with the exception of Fraction II had been decreasing after treatment with dexamethasone. No change was observed in the excretion of DHA even after the combined treatment with F.S.H.-H.C.G.

The level of androstenol increased to 320µg./24hr. on day 20. The greatest change observed however was in the excretion of Fraction II which increased after the treatment with F.S.H. and H.C.G. in a manner resembling that of pregnanediol. The ratio of II/III at day 16 was 6.5. By day 22 it had returned to almost the control level, which was 0.52. The increase in Fraction II suggested that there was an increase in the production of pregnanetriol by the ovary after stimulation with gonadotrophin.

Figure 33 - Excretion of 17-Oxosteroids.

The resultant effect of the treatment with dexamethasone and F.S.H. was to increase the level of excretion of 17-oxosteroids. On the other hand little





The effect of FSH and HCG on the excretion of DHA, androstenol, Fractions II and III, and 17-oxosteroids in patient G.

increase occurred after treatment with F.S.H. and H.C.G.

Conclusion:

The results of this preliminary experiment suggest that the doses of F.S.H, and F.S.H. and H.C.G. were adequate to cause stimulation of the biosynthetic pathways leading to oestrogen formation in the ovaries of this patient. Adequate suppression of adrenal steroids appeared to be maintained by the dose of dexamethasone used. Progesterone was detected in the luteal tissue and in the ovarian cyst fluid removed at laparotomy, and a freshly formed corpus luteum was also found.

Patient H.

The few estimations of urinary androstenol carried out in Patient G. suggested that dexamethasone failed to suppress completely, in contrast to DHA, the level of androstenol excreted. Both Patients H and I were treated with A.C.T.H. in order to obtain information about the hormone controlling the excretion of androstenol. Laparotomy was performed at different times during treatment with gonadotrophin.

Preliminary A.C.T.H. test:

A.C.T.H. was given to patients H and I (120i.u./day) for 2 days, before treatment with dexamethasone. The collections of urine were the same as for patient G.

Fig. 34. The excretion of oestriol and pregnanediol.

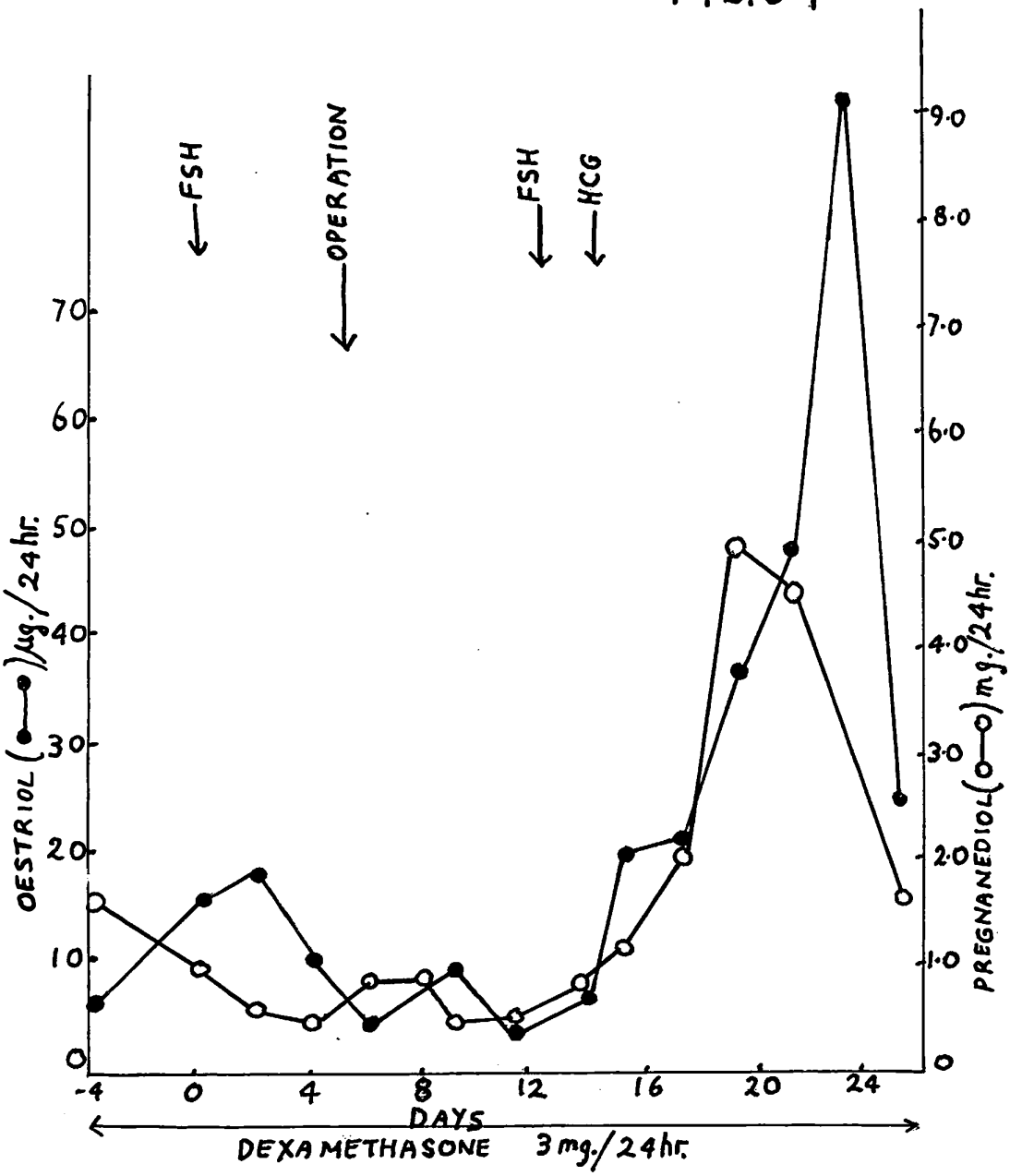
The treatment with A.C.T.H. had no effect on the excretion of oestriol but it increased that of pregnanediol from 1.8 mg./24hr. to 5.3 mg/24hr. The results in Figure 34 show that treatment with dexamethasone caused a small decrease in the level of pregnanediol but a threefold increase in the level of oestriol. Treatment with F.S.H. alone caused little change in either of the two steroid levels but after the combined treatment with F.S.H. and H.C.G. there was an immediate increase in the excretion of both steroids, the pregnanediol level reached 5.0mg./24hr. on day 18 and oestriol 92 µg./24hr. on day 22. The levels of both oestriol and pregnanediol increased and decreased at the same time. Laparotomy was performed on day 4.

Figure 35. The excretion of Fraction II and III.

The effect of A.C.T.H. on both Fractions II and III is shown graphically. The ratio of II/III for the control urine specimen was 0.34. After treatment with A.C.T.H. Fraction II increased to 8.5 mg. equivalents aetiocholanolone/24hr. and Fraction III to 29.4mg. equivalents aetiocholanolone/24hr. The ratio remained normal at 0.29.

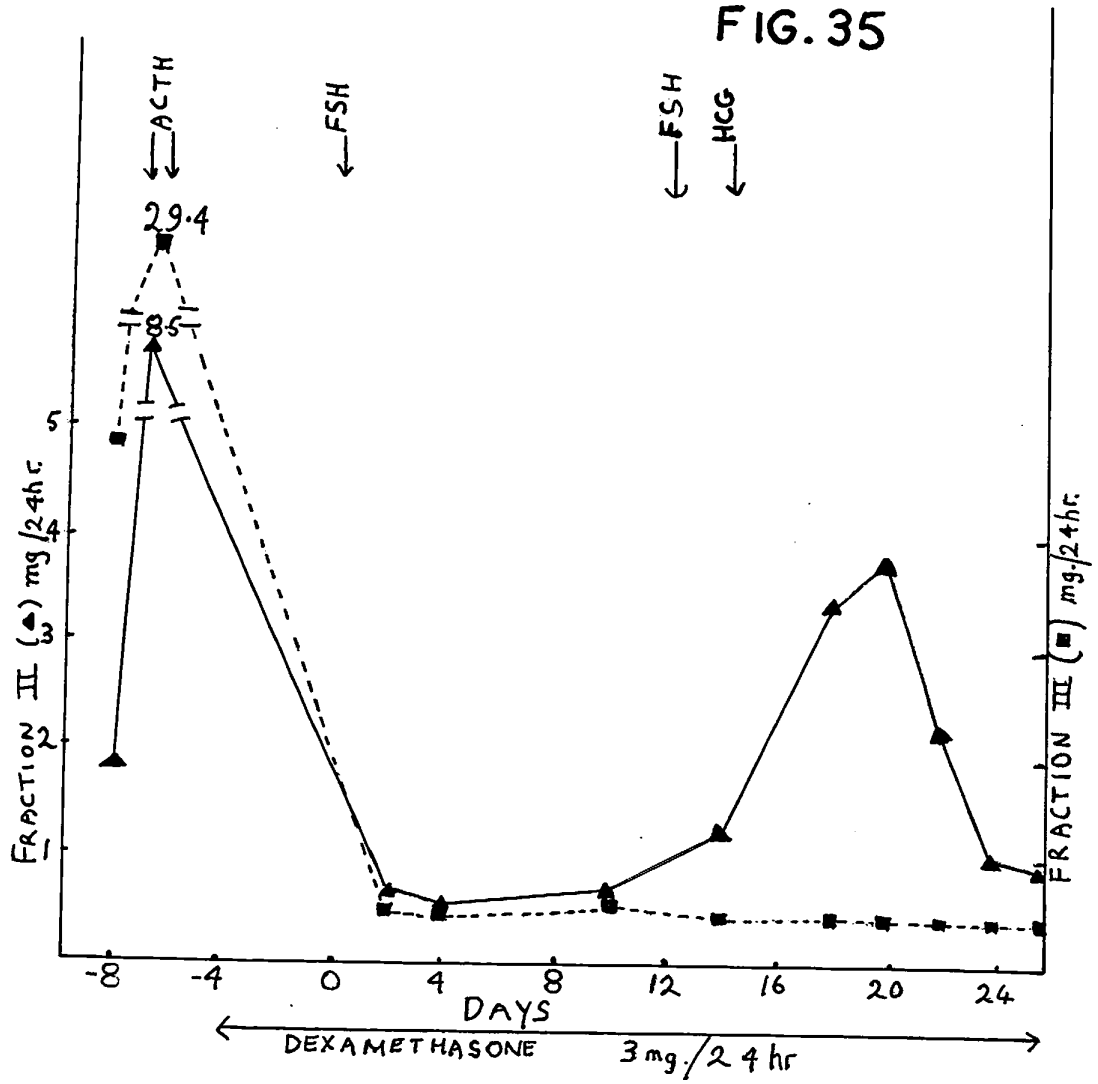
Treatment with dexamethasone decreased the excretion of both Fractions and a single injection of F.S.H. appeared to have no effect, and the excretion of Fraction III remained at this low level throughout the experiment. The combined F.S.H. and H.C.G. treatment caused an increase in Fraction II to 3.9 mg.

FIG. 34



The effect of FSH and HCG on the excretion of oestriol and pregnanediol in patient H.
The treatment with dexamethasone is also shown.

FIG. 35



The effect of ACTH, FSH and HCG on the excretion of Fractions II and III in patient H.
The treatment with dexamethasone is also shown.

equivalents aetiocholanolone/24hr. (Ratio 8.7), however the pattern of excretion of Fraction II was similar to that of pregnanediol (Figure 34). After day 20 the excretion of Fraction II decreased and by day 24 it was almost completely suppressed.

Figure 36. The excretion of dehydroepiandrosterone, androstenol and 17-oxosteroids.

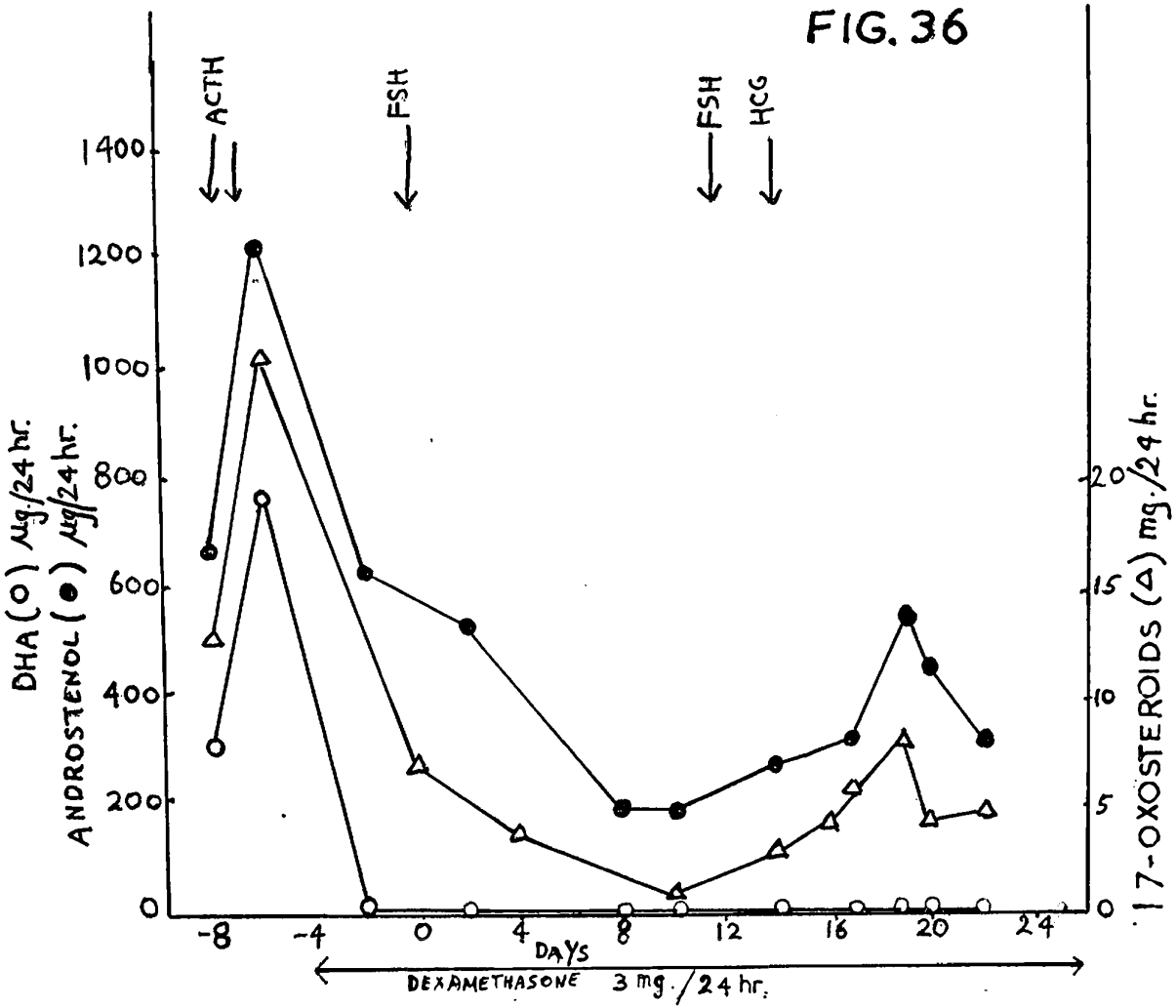
The levels of all three steroids increased after treatment with A.C.T.H. DHA increased from 360µg./24hr. to 780µg./24hr., androstenol from 640µg./24hr. to 1200µg./24hr. and 17-oxosteroids from 13.5mg./24hr. to 19mg./24hr.

Treatment with dexamethasone was followed by a decrease in each of the steroid levels, by day -2 the level of DHA in urine was not detectable and it remained undetected throughout the period of treatment. The effect of a single injection of F.S.H. was not clear as the excretion of 17-oxosteroids and androstenol had not reached their minimum levels after treatment with dexamethasone. By day 10 it was considered that complete suppression of the steroids had been achieved. At this point, however, the level of androstenol was 200µg./24hr. The effect of F.S.H. and H.C.G. was to increase the excretion of androstenol to a maximum of 525µg./24hr. on day 19. The excretion of 17-oxosteroids increased over the same period.

At laparotomy the ovaries were normal in size but contained numerous small cysts typical of the Stein-Leventhal Syndrome. Cyst fluid was taken from this patient and the results of steroid estimations are shown in Table VIII.

It was found that a freshly formed corpus luteum was present in one ovary, and that the cyst fluid contained progesterone, 17α hydroxyprogesterone, and an elevated level of androstenedione.

FIG. 36



The effect of ACTH, FSH and HCG on the excretion of DHA, androstenol and 17-oxosteroids. The treatment with dexamethasone is also shown.

Patient I.

The preliminary treatment with A.C.T.H. increased the excretion of oestriol from 3.0µg./24hr. to 7.0µg./24hr. and that of pregnanediol from 1.1mg./24hr. to 12.0mg./24hr.

Figure 37. The exretion of oestriol and pregnanediol.

Treatment with dexamethasone was followed by a threefold increase in the excretion of oestriol and a decrease in pregnanediol from 1mg./24hr. to 0.5mg./24hr. The single dose of F.S.H. had no effect on the pregnanediol but the oestriol increased to 25.0µg./24hr. on day 2. On day 8 it had fallen to 9.0µg./24hr. The effects of treatment with F.S.H. and H.C.G. was to increase the level of oestriol to 97.0µg./24hr. on day 14 and the excretion of pregnanediol from 0.7mg./24hr. to 1.7mg./24hr. On day 22 the levels of both steroids had returned to those observed before treatment with gonadotrophin.

Figure 38. The excretion of androstenol and dehydroepiandrosterone.

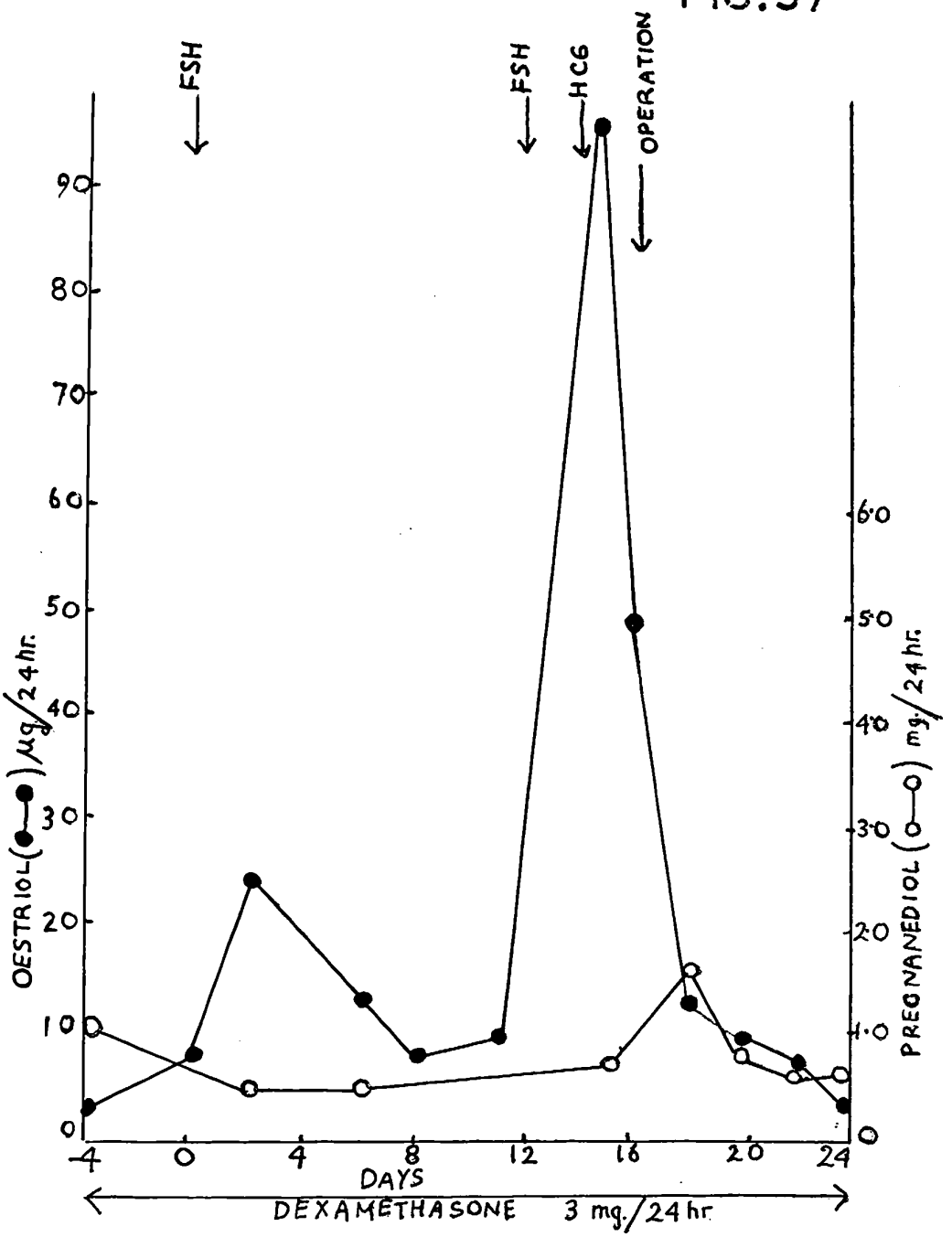
The effect of A.C.T.H. on the levels of androstenol and DHA is shown. After treatment with A.C.T.H. the level of androstenol increased from 890µg./24hr. to 3900.0µg/24hr. DHA was increased, but to a lesser degree, after the same treatment. Treatment with dexamethasone caused a complete suppression of both DHA and androstenol on day 6. The effect of F.S.H. alone was obscurred by the fact that the steroid levels were falling after treatment with dexamethasone. The combined F.S.H. and H.C.G. treatment brought about an increase in androstenol from 0µg./24hr. to 300µg./24hr. on day 20. No change was observed in the excretion of DHA.

Figure 39. Excretion of 17-oxosteroids and 17-oxogenic steroids.

This graph shows the response of 17-oxo and 17-oxogenic steroids to treatment with A.C.T.H. Treatment with dexamethasone suppressed the levels of both steroids but a slight increase in excretion of 17-oxosteroids was observed after treatment with F.S.H. The combined F.S.H. and H.C.G. treatment had no effect on the 17-oxosteroid.level but a slight increase in 17-oxogenic steroids was observed.

On day 16 laparotomy was performed and the ovaries of this woman were small in size and contained many

FIG.37



The effect of FSH and HCG on the excretion of oestriol and pregnanediol in patient I.
The treatment with dexamethasone is also shown.

FIG.38

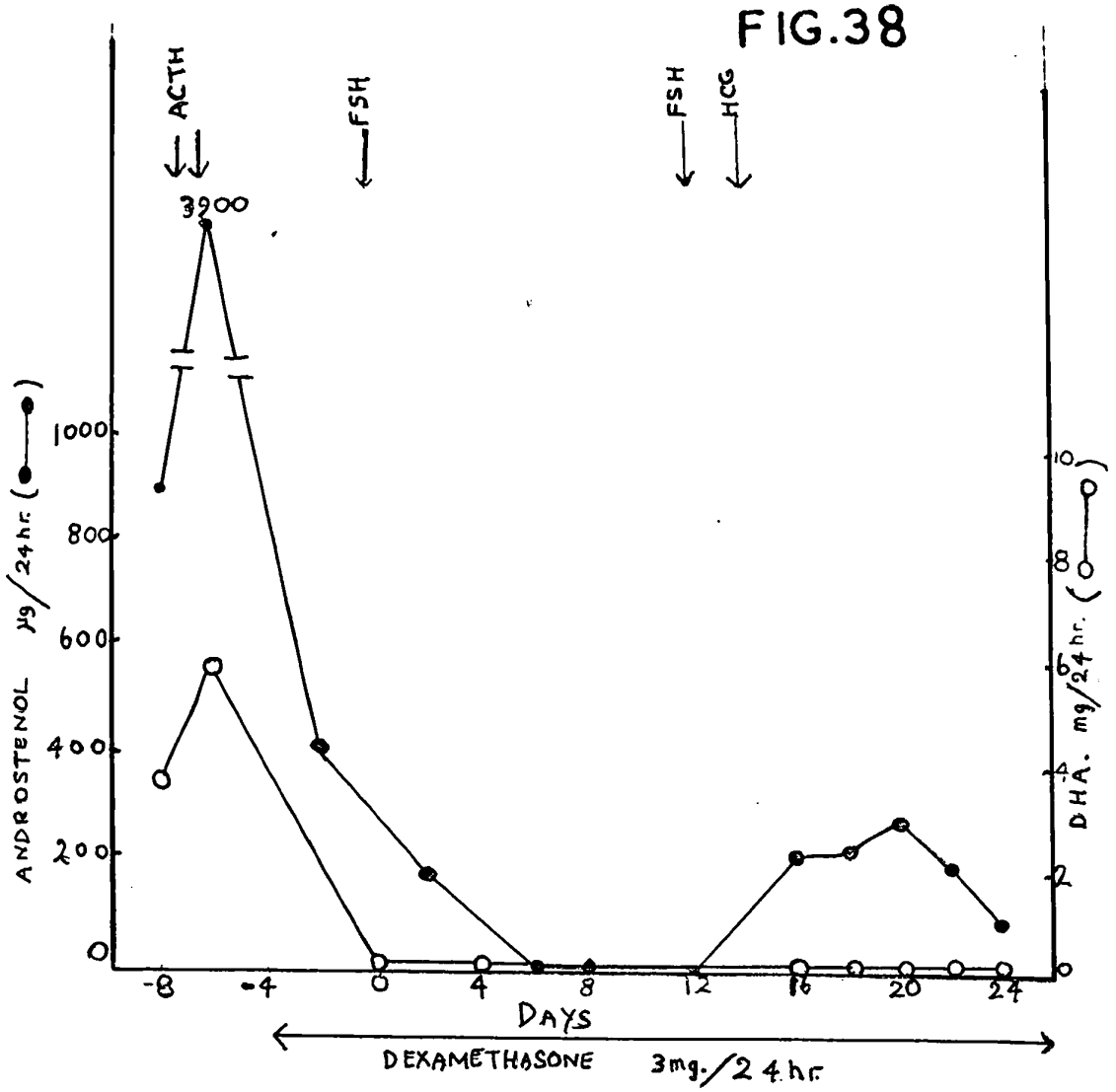
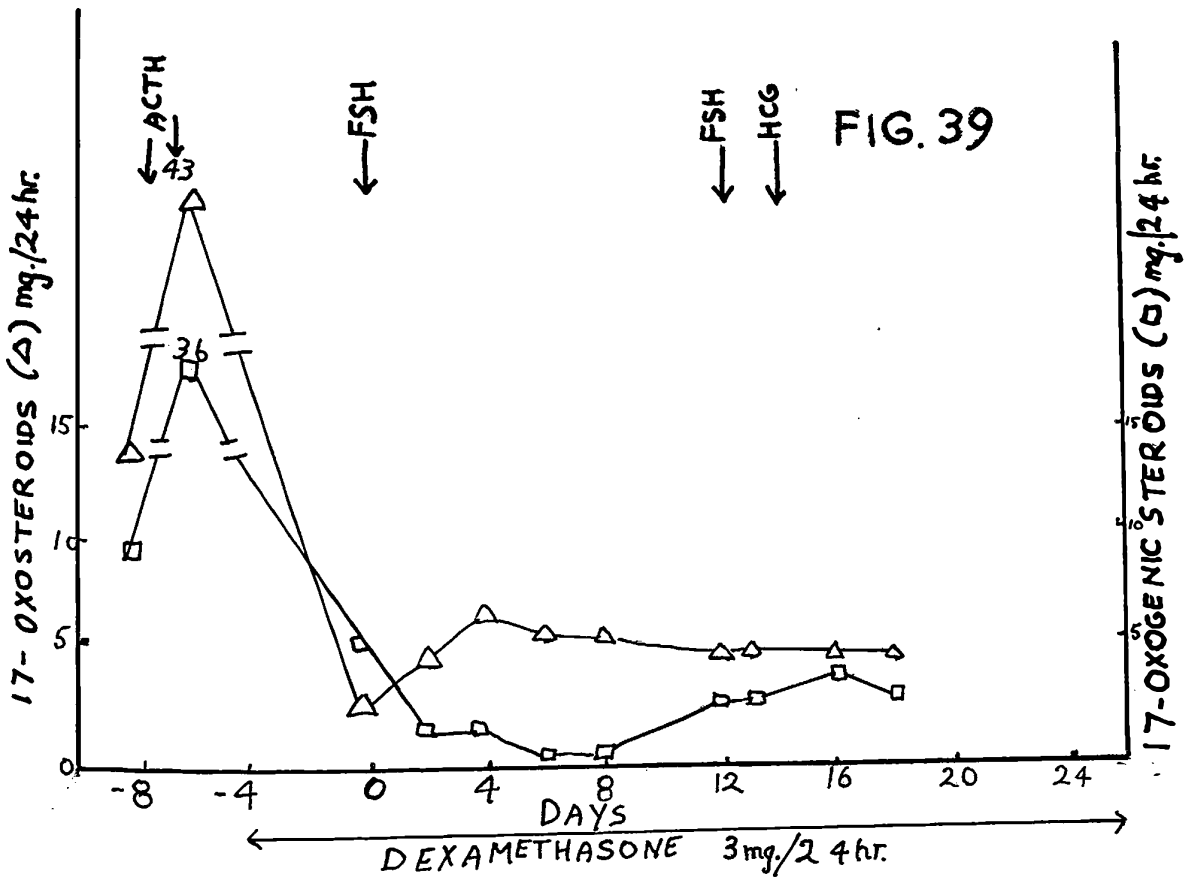


FIG. 39



The effects of ACTH, FSH and HCG on the excretion of DHA, androstenol, 17-oxogenic and 17-oxosteroids.

small cysts, no corpus luteum was found. Ovarian cyst fluid was removed and the results are shown in Table VIII. This woman had elevated levels of 17-α-hydroxyprogesterone and androstenedione.

Conclusion:

In these two patients (H and I) the excretion of androstenol was controlled by A.C.T.H. The doses of gonadotrophin used had no effect on the excretion of DHA but caused an increase in the level of urinary androstenol in both women. A freshly formed corpus luteum was found in the ovary of one patient (patient H).

The follicular fluid taken at different times during treatment showed no change in steroid content from that found in the untreated woman with Stein-Leventhal Syndrome. In both women dexamethasone increased the level of urinary oestriol.

The results of patients H and I had shown that the excretion of oestriol started to decrease after a single injection of F.S.H. and that the greatest change in the excretion of both oestriol and pregnanediol occurred after the combined F.S.H. and H.C.G. treatment. In the next five patients the interval between the 2 doses of F.S.H. was reduced to 4 days and dexamethasone was omitted. The A.C.T.H. test was also omitted as it had been established that the excretion of androstenol was under the influence of this hormone. Urine collections and presentation of results were the same as described for Patients G, H. and I.

Gonadotrophins Used:

Each of the next five patients was treated with 2 injections of F.S.H. (2,000 mg./equivalents/1RP-HMG/ day). There was a four day interval between the 2 injections, and the second injection of F.S.H. was followed 2 days later by H.C.G. (12,000 i.u.) in a single injection.

The results and treatment schedules are shown graphically.

Patient J:

Figure 40a: The excretion of oestriol and pregnanediol.

The single injection of F.S.H. was followed by an increase in oestriol from 8.0µg./24hr. to 40µg./24hr. on day 4 but only a slight increase in that of pregnanediol. The combined treatment with F.S.H. and H.C.G. increased the excretion of oestriol steadily to 486.0µg./24hr. and that of pregnanediol to 14.4mg./24hr. on day 12. On day 12 laparotomy was performed.

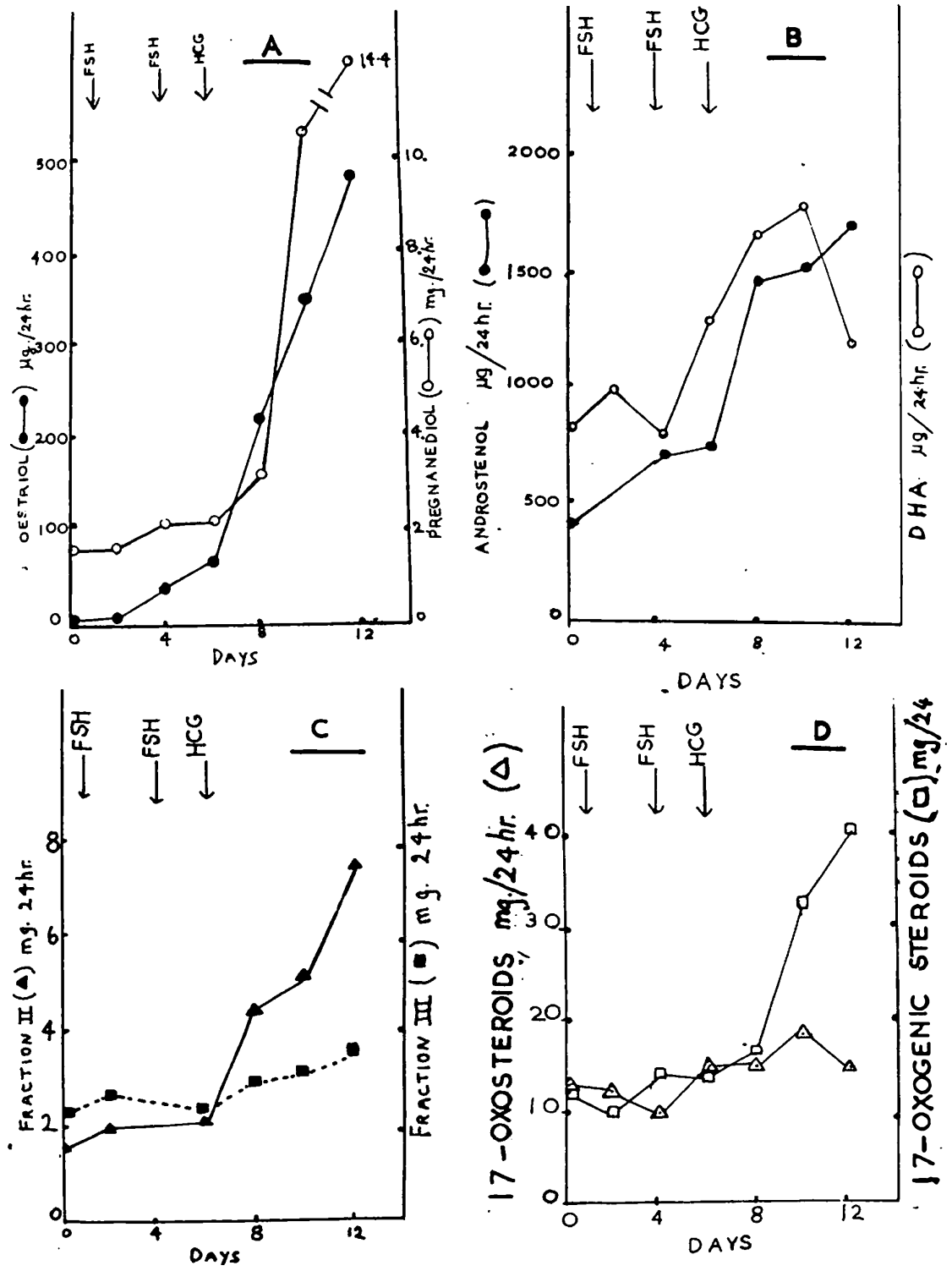
Figure 40b: The excretion of androstenol and dehydroepiandrosterone.

The single injection of F.S.H. caused increases in the excretion of both androstenol and DHA, but after the combined treatment with F.S.H. and H.C.G. androstenol increased from 786µg./24hr. on day 6 to 1720µg./24hr. on day 12. Over the same period the excretion of DHA increased from 800µg./24hr. to 1800µg./24hr. on day 10, but in contrast to the level of androstenol it had begun to fall on day 12.

Figure 40c: The excretion of Fraction II and Fraction III.

The changes in both fractions are shown graphically, the ratio of both fractions on control day was 0.67.

FIG. 40



The effect of FSH and HCG on the excretion of :-
(A) oestriol and pregnanediol
(B) androstenol and DHA
(C) Fractions II and III
(D) 17-oxosteroids and 17-oxogenic steroids
in patient J.

The single injection of F.S.H. had no effect on either Fraction II or III but the combined treatment with F.S.H. and H.C.G. brought about a large increase in Fraction II from 1.6mg. to 7.7mg. equivalents of aetiocholanolone/24hr. and Fraction III increased from 2.4mg. to 3.7mg. equivalents of aetiocholanolone/24hr. Over this period the ratio increased steadily reaching 2.08 on day 12. In these patients (J and K) the increases in the ratio are not as large as in the first 3 patients where the difference between fractions was magnified by the suppression of Fraction III. On day 12 the levels were still elevated.

Figure 40d: Excretion of 17-oxosteroids and 17-oxogenic steroids.

The pattern of excretion of 17-oxosteroids and 17-oxogenic steroids is shown: The single dose of F.S.H. had little effect on either the 17-oxo or the 17-oxogenic steroid levels but the combined F.S.H. and H.C.G. increased the 17-oxosteroids from 10mg./24hr. to 18mg./24hr. on day 10. Over the same period the 17-oxogenic steroids increased from 14mg./24hr. to 41mg./24hr.

At laparotomy the ovarian tissue taken from the patient showed extensive haemorrhage both into the cysts and into the ovarian tissue. Small and large cysts were present in the ovaries and some of the cysts showed luteinised granulosa cells.

Ovarian cyst fluid removed at laparotomy contained a large quantity of progesterone, 17α hydroxyprogesterone, oestradiol-17β, oestrone and a smaller amount of androstenedione. The results are shown in Table VIII.

Patient K:

This patient was treated in an identical manner to Patient J.

Results are shown graphically in Figure 41a,b,c,d.

Figure 41a: Excretion of oestriol and pregnanediol.

The control level of excretion of oestriol (26.0µg./24hr.) was higher than that normally encountered in women with the Stein-Leventhal Syndrome. The excretion of pregnanediol however was within the normal limits. The single injection of F.S.H. increased the excretion of both steroids. The greatest change was observed in the excretion of oestriol after the combined F.S.H. and H.C.G. treatment while that of pregnanediol increased steadily from the beginning of treatment. The excretion of pregnanediol had risen to 9.4mg./24hr. on day 12 but that of oestriol reached a maximum of 200µg./24hr. on day 10 and had fallen to 120µg./24hr. on day 12. Laparotomy was performed on day 12.

Figure 41b: Excretion of androstenol and dehydroepiandrosterone.

After a single injection of F.S.H. the excretion of DHA decreased slightly but that of androstenol had increased from 306µg./24hr. to 550µg./24hr. After the combined F.S.H. and H.C.G. treatment the DHA level increased from 770µg./24hr. on day 4 to 3,000µg./24hr. on day 12 while that of androstenol increased steadily from a control value of 306µg./24hr. to 1,190µg./24hr. on day 10.

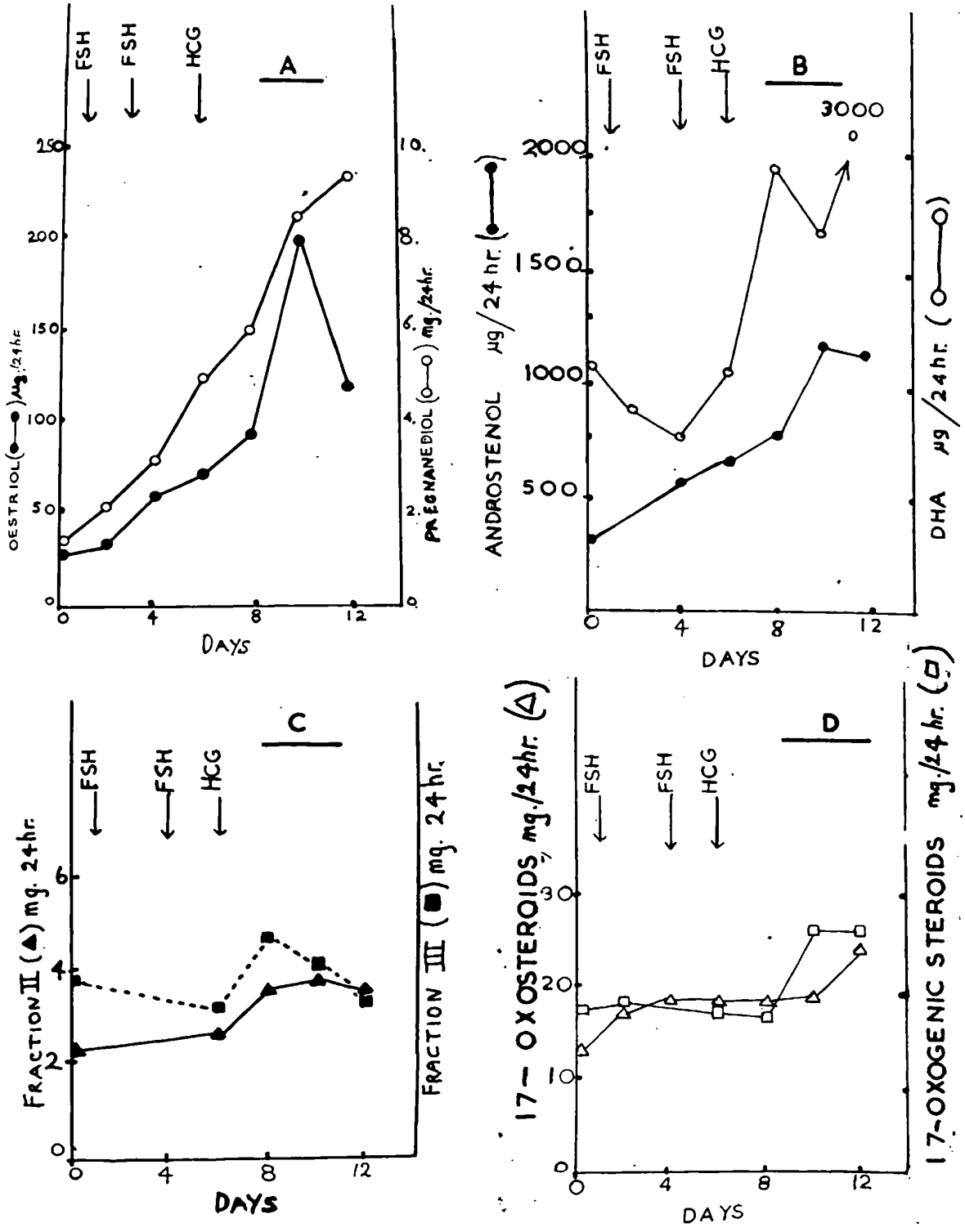
Figure 41c: Excretion of Fractions II and III.

The control ratio of Fraction II/III was 0.61. A single dose of F.S.H. had little effect on either of the two fractions, on the other hand after the combined F.S.H. and H.C.G. treatment there was a slight increase in both fractions and the ratio increased steadily to 1.05 on day 12.

Figure 41d: Excretion of 17-oxosteroids and 17-oxogenic Steroids.

The single injection of F.S.H. caused a small increase in the excretion of 17-oxosteroids but there was no change in 17-oxogenic steroids. After the combined F.S.H. and H.C.G. treatment the 17-oxogenic steroid level increased from 18mg. to 24mg./24hr. on day 8 and the 17-oxosteroid level increased from 19mg./24hr to 23mg./

FIG. 41



The effect of FSH and HCG on the excretion of :-
(A) oestriol and pregnanediol
(B) androstenol and DHA
(C) Fractions II and III
(D) 17-oxosteroids and 17-oxogenic steroids
in patient K.

24hr. on day 12. Laparotomy was performed on day 12.

The ovaries of the patient were found to be enlarged and similar to those of patient I, but a recent corpus luteum was found on the right ovary. The cyst fluid from both the left and right ovaries contained large quantities of progesterone, 17α hydroxy progesterone, and increased quantities of oestradiol -17β and oestrone. Androstenedione and pregnenolone were also found.

Results are summarised in Table VIII.

Conclusion:

Both of the patients showed similar responses to gonadotrophin. The effect of shortening the time interval between the injections of F.S.H. appeared to be more effective as judged by the marked increases found in the excretion of oestriol and pregnanediol. This also had the effect of increasing the level of DHA in urine, this was not found in the previous three patients G, H. and I. The level of androstenol also increased. The follicular fluid contained large quantities of progesterone, 17α hydroxyprogesterone, and oestradiol-17β but low levels of androstenedione. This suggested that treatment with gonadotrophin had altered in some way the metabolism and biosynthesis of the steroid present in ovarian follicular fluid.

Table V111 shows the concentration of steroids in fluid from cysts and in luteal tissue.

Cyst fluid - Results as µg./100ml.

Patient.	Vol.ml.	Pregnenolone.	17αOH Pregnenolone.	D.H.A.	Progesterone.	17αOH Prgesterone.	Androstenedione.	Oestrone.	Oestradiol-17B	Oestriol.	19-OH androstenedione.	20-OH Pregn-4-en-3-one.
G	1.06	-	-	-	<10	<10	<19	<5	<5	-	-	-
H	0.8	-	-	-	12	12	63.0	<6	<6	-	-	-
I	3.0	<1.5	-	<1.5	5	88.5	130	<2	<2	-	-	-
J	10.0	<0.5	<0.5	<0.5	348	102	12	2	34	0.5	<10	<10
K	20.0	0.5	<0.3	<0.3	550	124	22	1	17	0.3	<5	5.5
K	12.0	0.8	<0.4	<0.4	592	148	18.4	3.8	15.4	-	<8	<8
					Luteal tissue - Results as µg./100g.							
G	1.49	-	-	-	23.5	<7	<7	<7	<7	-	-	<7

The results of the steroid estimations in follicular fluid from all five patients show that an elevated level of androstenedione occurred when the levels of progesterone, 17α hydroxy progesterone and oestradiol-17β were low. In patients J and K in contrast, androstenedione was low when the other steroids were elevated. Some luteal tissue removed from Patient G contained progesterone.

In the next two Patients (L and M) treatment with gonadotrophin was given in a similar manner as in Patients J and K but urine specimens were collected at longer intervals after treatment. Steroid estimations were carried out on single 24hr. specimens of urine collected on the days indicated. Similarly laparotomy was performed after a longer interval from the beginning of treatment.

Patient L.

RESULTS

Days after 1st F.S.H. treatment.	17-oxo-steroids mg./24hr.	17-oxogenic steroids mg./24hr.	Oestriol µg./24hr.	Androstenol µg./24hr.	Pregnanediol mg./24hr.
Control	12	20	9.0	210	0.8
9	27	62	490	973	13.4
16	-	40	1120	-	17.9
17	15	26	1210	351	8.9
24	12	22	16	230	0.8
40	Laparotomy				

Ovarian cyst fluid taken at laparotomy was collected in labelled tubes from individual cysts. The fluid from 4 small cysts was pooled 1 & 2, 3 & 4, but the others were estimated on fluid from individual cysts. Results are shown in Table IX.

In this patient a similar type of response was obtained as in Patients J and K, where large increases in oestriol, pregnanediol, and androstenol were obtained. DHA was not estimated in this patient but the 17-oxosteroids and androstenol and the level of 17-oxogenic steroids had increased. On day 24 the steroid levels had returned almost to control values. Over the period of

Table 1X Shows the steroids estimated in fluid taken from different cysts at laparotomy on Patient L.

Cyst fluid - Results as ug./100ml. of fluid.

Individual Cysts.	Pregnelonone.	Progesterone.	17 α Hydroxy Pregnenolone.	D.H.A.	17 α Hydroxyl Progesterone.	Androstenedione.	Oestrone.	Oestradiol-17B
1 & 2	<1	<4	<1	5.6	18.9	69	2.2	2.2
3 & 4	<2.2	<1.7	<0.4	24.8	10.4	55.5	<0.9	<0.9
5	<1.5	<2	<0.5	17	16.5	71	<1.0	<1.0
6	<1.5	<6	<1.5	7.4	26.5	121	<1.5	<1.5
7	<2.6	<1.8	<2.6	25.1	18.3	75.8	<0.9	<0.9
8	<0.95	<2.0	<0.4	11.4	19.0	54.2	<0.5	<0.5
9	<2.0	<3.0	<0.5	23.5	22.0	77	<0.5	<1.0

increased oestrogen output gross enlargement of the ovaries occurred.

The steroids present in follicular fluid showed the same constituents in each of the individual cysts but a distinctly different pattern to that previously found in the patients G,H,I,J and K. In this patient the progesterone level was low but the androstenedione and DHA levels were elevated. This had been the first time that DHA was detectable in the cyst fluid of any of the patients studied. Laparotomy was performed on day 40.

The portion of ovary removed at laparotomy contained large follicular cysts and massive oedema. The appearances were consistent with acute enlargement of the ovary due to hormone stimulation.

Patient M.

This patient was treated in an identical manner to Patient L and steroid estimations were done on the days shown below.

RESULTS

Days after start of F.S.H. treatment	17-oxo steroids. mg./24hr.	17-oxogenic steroids. mg./24hr.	Androstenol µg./24hr.	Oestriol µg./24hr.	Pregnanediol mg./24hr.
Control	13	13	192.0	9.9	1.1
9	24	31	1080.0	430.0	9.5
23	16	18	226.0		1.1
96	Laparotomy.				

At laparotomy cyst fluid was taken from both the left and right ovaries. Results are shown in Table X.

	Value of fluid.	Pregnenolone	17-OH Progesterone.	Progesterone.	17-OH Progesterone.	Androstenedione.
Left Ovary	1.7	3	3	8.8	23.5	208
Right Ovary	.65	8	8	30.8	30.8	262

Results are expressed as µg/100 ml. of cyst fluid

The steroids present in the follicular fluid removed at laparotomy from patient M are shown.

The response to gonadotrophin treatment was similar in this patient to the type of response found in patient J, K and L where increased levels of oestriol, pregnanediol and androstenol occurred. The cyst fluid removed at laparotomy contained elevated levels of androstenedione and low levels of progesterone and 17-hydroxyprogesterone.

The portion of the right ovary removed at laparotomy showed, histologically, a thin layer of condensed cortical stroma beneath which there were many small cysts. Most of the cysts were lined by granulosa cells and for the most part there was a well defined and sometimes very prominent layer of theca interna. A corpus luteum was observed in the left ovary.

Patient N.

This patient was treated as Patients L and M and urine analysis was performed on the days shown. Since little response was obtained in the first month of treatment on the day selected (day 9), the treatment was repeated to find out if the effects of the treatment were cumulative. In the third month the level of H.C.G. was increased to 24,000 i.u./day.

Results are shown:

First month.

Days after start of F.S.H. treatment.	Oestriol µg./24hr.	Pregnanediol mg./24hr.
Control	8.2	1.0
9	23.8	1.1
Second month.		
9	34.0	1.3
11	23.3	1.2
18	17.0	1.1
Third month.		
9	17.0	1.2
12	9.4	1.5
13		1.0
67 Laparotomy.		

At laparotomy cyst fluid was collected from cysts on the left and right ovaries. Results are shown in Table XI.

Table XI

RESULTS.

	Vol.	Progesterone.	17-OH Progesterone.	Androst-enedione.	Oestrone.	Oestradiol.
L. Ovary	1.5	17.0	29.2	117	4	4
R. Ovary	1.2	13.	13	67	3	3

Table XI. The steroids in the follicular fluid removed at laparotomy from Patient N are shown. Results are expressed as ug./100ml.

These results show that this woman responded differently from Patients J, K, L and M and in fact failed to respond any more even to elevated H.C.G. levels.

The androstenedione level of the follicular fluid was elevated which is the constituent found consistently in the ovaries of the untreated Stein-Leventhal Syndrome.

Conclusion:

a) Treatment with gonadotrophin was followed by increased excretion of oestriol, pregnanediol, 17-oxo and 17-oxogenic steroids in two of the patients. b) One patient did not respond during two successive months and failed again with an increased level of H.C.G. c) Steroids in individual cysts appeared to contain the same constituents but DHA was measured in the cyst fluid from one patient. d) The reversal of the steroid pattern in the follicular cyst fluid to that found in untreated women with the Stein-Leventhal Syndrome suggested that the changes brought about by gonadotrophin were of a reversible nature.

DISCUSSION

DISCUSSION

In the development of a chemical method four factors are of importance (Borth, 1952; Loraine, 1958) (i) it is necessary to know how near an analytical procedure approaches the true result (accuracy) (ii) the reproducibility of the results (precision) (iii) the least amount that is distinguishable from zero (sensitivity) (iv) and whether the method measures what it is claimed to measure (specificity).

Since the amount of oestriol in a given quantity of urine cannot be known with certainty, the accuracy of the method was investigated by recovery experiments. These experiments were performed by adding oestriol to acid hydrolysed urine, thus the stage of hydrolysis was not examined although it is known that losses occur at this point. A further disadvantage is that at low levels of added oestriol the error in estimating the endogeneous oestriol present may be large by comparison with the quantity added, so that the results at very low levels of oestriol become meaningless. By adding oestriol to acid hydrolysed urine the accuracy of the short method was investigated. The mean percentage recovery over the range 4.5 - 40.0 µg./24hr. was between 76 - 87%, at the low levels, the mean percentage S.D. was greatest but comparable with the results published by Brown (1955) and Brown, Bulbrook & Greenwood (1957a).

Precision:

The precision of the short method was derived from the differences between duplicate estimations over a wide range of levels of oestriol. The standard deviation of the method of Brown was derived over the range recommended by that worker and it was found that there was very little difference between the standard deviations of the two methods. In fact the standard deviation of the short method in the range 4.5 - 9.9µg./24hr. was considerably lower than that in the method of Brown. The standard deviation of the short method increased as the concentration of oestriol increased but it was not possible to establish any correlation between the level of oestriol and the standard deviation at that level.

Sensitivity:

The lower limit of 5.0µg./24hr. of oestriol was originally chosen by Brown (1955) as the lower limit of

the estimations since below this the error becomes comparatively large and the values of less than 5.0 µg/24hr. were considered to be of little significant importance. A later modification of the method by Salokangas & Bulbrook, (1961) enabled much smaller quantities of oestriol to be estimated with confidence. In the method described here a lower limit of 4.5µg/24hr. was arbitarily chosen for normal routine purposes but smaller quantities can be estimated by reducing the volume of chloroform used for the extraction of the Kober colour and by using micro-cuvettes for colorimetry.

Specificity:

Conclusive evidence for the specificity of a quantitative chemical estimation is difficult to obtain. The best that can be done is to accumulate as much evidence as possible to show that the method measures what it is supposed to and nothing else. The specificity of the original method of Brown, (1955) has been checked by many workers (Brown, 1955; Diczfalusy, 1955; Bauld, 1956; Diczfalusy & Westman, 1956; Brown et al, 1957 a,b; Gallagher, Kraychay, Fishman, Brown & Marrian, 1958; Brown & Blair, 1960; Aldercreutz, Diczfalusy & Engstrom 1960; Fishman & Brown, 1962). A later modification of the method by Salokangas & Bulbrook (1961) established the specificity of the colour extraction procedure.

The specificity of the short method is established on the basis that it includes all of the stages on which the specificity of the method of Brown (1955) was based. Also it included a further step of colour extraction into chloroform with the characteristic change in the absorption spectrum of the extracted Kober colour of oestriol methyl ether. The only stage of the short method which differed from that of Brown (1955) was the partition of oestriol between benzene/petroleum ether/water. This step was replaced by extraction of oestriol from diethyl ether by dilute alkali and this procedure is used on the modification by Brown et al (1957 b).

Comparison of the two methods suggested that the short method was suitable for routine use. It is known that some loss of oestriol occurs during acid hydrolysis and that this is due to the pigment present rather than to the strength of the acid used for hydrolysis (Brown & Blair, 1958). The same loss of oestriol during hydrolysis probably occurs in the short

method also. However, this method is suitable for following the pattern of oestriol excretion during the normal menstrual cycle and for the estimation of oestriol in the urine of pregnant women. The effects of the drugs (Brown et al. 1957 b) which interfere with the estimation of oestriol were not investigated. Since the experimental work on the short method was completed another method for the estimation of oestriol has been published by Brown & Coyle (1963), this procedure is almost identical to the short method described here except that the colour extraction stage was omitted. In this method the smallest amount of oestriol that was detected was 17.0 µg./24hr. and at the level of 70.0 µg./24hr. the mean percentage standard deviation was ± 25%. These workers compared the method with the earlier method of Brown (1955) and found that it was suitable for the estimation of oestriol during pregnancy.

The main advantage of the method described in this work is that more estimations can be performed during a day, it is possible to perform ten estimations compared with four estimations by the method of Brown (1955). The method is now being used to study the response in oestriol levels in women with amenorrhoea treated with gonadotrophic hormones and to study the excretion of oestriol during the pregnancies which followed treatment in some of the patients.

Clinical Trial: Treatment of women with secondary amenorrhoea using human gonadotrophin preparations:

In the present studies several preparations of human gonadotrophins have been given in different ways to a small number of patients with secondary amenorrhoea in experiments designed so that the results could be analysed statistically. The enabled a large amount of detailed information to be obtained about these preparations and methods for their administration from a limited amount of material.

The patients had amenorrhoea of long-standing. They had all been referred after thorough gynaecological examination and empirical treatment with various hormones elsewhere. They had then been under observation in the Department of Clinical Endocrinology for more than a year during which time they had had further treatment. This aimed at establishing whether they could be induced to menstruate by suggestion. They were given either 0.5mg. stilboestrol daily for 14 days each month for three consecutive months or biologically inert tablets made by British Drug Houses Ltd., to resemble the tablets of stilboestrol. For 12 months they received three courses of three months treatment each of the control tablets and one of stilboestrol. The first and last courses were of control tablets but the middle two were randomly arranged without the order being known to the consultant in charge. None of the patients menstruated spontaneously during this time. Shortly before they began treatment with gonadotrophins they were re-examined and all were found to have atrophied uteri and ovaries with excretion of urinary gonadotrophins in the lower half of the normal range, but they had no other evidence of organic disease. They were considered to have a bad prognosis with regard to spontaneous menstruation and fertility but, finally, each patient received control injections of gelatin, instead of F.S.H, during one month in the first experiment and again failed to respond. These patients have, therefore, been regarded as a homogeneous sample of the same population and it is likely that a larger series of similar patients would respond in a like manner.

The patterns of excretion of oestriol and pregnanediol induced by treatment have been compared with the mean patterns of excretion in normal women

determined by the same biochemical methods. The normal pattern for oestriol described by Brown (1955) showed a gradual rise to a peak at mid-cycle followed by an abrupt fall. There was a more prolonged secondary rise and slower fall during the luteal phase (Figure 2A and B). It is difficult to assess the variation in the excretion of oestriol amongst these normal women since Brown only quoted the maximum, mean and minimum figures. Perusal of these charts show, however, that there was little rise in the mean response during the luteal phase although there was a considerable rise in the maximum response. This suggests that only a few of his subjects showed much increase in the excretion of oestriol at this phase of the cycle. The normal pattern for excretion of pregnanediol described by Klopper (1957) (Figure 3A, B and C) is simpler than that for oestriol and its presence or absence is obvious during the luteal phase of the cycle.

Inspection of the results of the individual trials in each Experiment show several different patterns of excretion. Examples of a single rise of oestriol occurring at mid-cycle can be seen in Figure 4, A3: 9, B6 and 14,E12, and of a rise at mid-cycle followed by a secondary rise in Figure 8, A5 and 14, B12. These are presumed to have been associated with ovulation because there was a rise in excretion of pregnanediol during the luteal phase of the cycle. Double rises in the excretion of oestriol are seen in an exaggerated form in Figure 10, D6 and D8. The latter show more clearly that the abrupt fall in excretion of oestriol immediately after the mid-cycle peak coincides with a rise in excretion of pregnanediol. It reflects the sudden change in steroid synthesis from oestrogen to progesterone and is presumably associated with the rupture of a follicle. The large secondary rise in oestriol is probably derived from other follicles and may indicate multiple ovulation. This is supported by the fact that patient D became pregnant in the month during which her figures for steroid excretion followed the pattern shown in Figure 10, D8, and she subsequently gave birth to dissimilar twins. It is also known that the luteal tissue present after ovulation has occurred is capable of secreting oestrogen, this is supported by the finding of sustained oestriol levels when conception occurred in each of the four patients. Oestradiol-17β and oestrone have been

detected in extracts of human corpora lutea (Zander, Brendle, von Munstermann, Diczfalusy, Martinsen and Tillinger, 1959). A very large secondary rise in excretion of oestriol is therefore probably undesirable.

An abnormal pattern of excretion of oestriol is shown in Figure 9, A7 and B8. Here there is a mid-cycle peak without a rise in excretion of pregnanediol and it probably represents the development of a follicle which failed to rupture. Another type is seen in Figure 8, B5. Here there is no mid-cycle peak of oestriol but there is a broad secondary rise which is again unassociated with a rise in excretion of pregnanediol and presumably also represents a failure to ovulate. Brown and Matthew (1962) described similar patterns in cystic glandular hyperplasia. These are all unsatisfactory responses and indicate unsuitable stimulation with gonadotrophins. Occasionally the peak in excretion of oestriol occurs abnormally early as shown in Figure 14, B13. This might be a delayed response to the previous month's treatment.

Only patient C showed an abnormally high output of pregnanediol in the control period (Figure 4,C3). It tended to fall to normal on treatment with follicle stimulating hormone (Figure 4, C1 and C2).

The magnitude of the responses in some of the trials in Experiments 2 and 3 tended to obscure the effects of others in the same experiments. The excretion of oestriol and pregnanediol by patient D, shown in Figure 9, D6 and D8, and of pregnanediol by Patient B in Figure 14, B12 are examples of such responses. They were, therefore, omitted from the analysis of variance so that deductions could be made from the remaining results. Since the figures for the excretion of the two steroids were analysed separately it was felt that important information about their relationship to one another, both in magnitude and timing, may have been neglected. The responses in each trial were compared directly to the steroid changes in normal cycles and scores accorded to (a) increases in oestriol in the ovulatory phase (b) increases in pregnanediol in the luteal phase (c) the relative positions of each peak (d) for the amount of increase obtained in each peak and (e) the length of time that the levels remained elevated. Analysis of these scores agreed with the results of the statistical analysis and did not add any further information.

Two kinds of experimental error could be estimated from the trials. The first kind was due to errors in collection and measurement and to short-term day to day fluctuations and could be estimated from comparisons of consecutive samples within each four-day period. It appears in the Analysis of Variance as the Residual Error. It is probable that this error variance increased with the magnitude of the response and theoretically a transformation of the measurements should have been made to equalize the variances. A small number of analyses of variance were therefore made on the logarithms of the results. The deduction from these analyses differed in no way from those made previously, but the confidence limits were a little wider and asymmetrical.

The second kind of error was due to the inconsistency of responses in the same patient to an identical treatment. Gemzell et al. (1960) claimed that the effect of F.S.H. on the ovarian size and the excretion of oestrogen did not change from one course of treatment to another identical one. Without replication this error could not be estimated specifically in this series of experiments but it was estimated by the analysis of variance and is shown for Experiments 1 and 2 as "Residual/Square" and as "Interaction" in Experiment 3. This interaction could be sub-divided into six components each attributable to the inter-dependence of two of the treatment factors. The significance of each of these interdependences was used to make inferences about the method of treatment rather than to measure the experiment error. Since these inferences were based on one experimental trial, without replication, they will need to be reconsidered in further experiments. The first experiment showed a difference in sensitivity between patients to a given dose of F.S.H.

Analysis of the figures for oestriol for the four patients failed to demonstrate any difference in response from month to month. The figures for pregnanediol for patients A, B and D were therefore analysed by use of the Youden Square to investigate a possible monthly variation. Patient C was excluded from this analysis because of her abnormally high figures throughout. The analysis indicated that the response to treatment during the first two months was lower than that in the third and fourth months, but that the response in the fourth month

was in fact lower than that in the third month. This suggested that with the low dosage of F.S.H. used there might be a build-up in response which was complete by the end of the third month.

The response to the different preparations of gonadotrophin was surprising. Each was given in the same dosage measured as F.S.H. but they differed widely in their content of luteinizing hormone. It was anticipated that the preparation with the highest concentration of luteinizing hormone and therefore greatest total amount of gonadotrophin would be the most effective. Moreover it seemed likely that the two hormones in this preparation would have the greatest joint action but the results showed that there was little difference between preparations, and, if anything, the one with the least amount of luteinizing hormone was the best.

An attempt was made in the next experiment to avoid abnormally high excretion of oestriol in the latter half of the cycle since, as suggested earlier, this might indicate the development of an excessive number of follicles. It was based on some preliminary data published by P.S. Brown (1959) which suggested that the excretion of F.S.H. is high at the beginning of the normal cycle and falls towards the end. Moreover, Buxton and Herrmann (1961) had shown at operation that treatment with F.S.H and H.C.G. given together in constant daily dosages had stimulated the production of multiple follicles and corpora lutea, but that these were of surprisingly different ages. It was thought therefore that an initial big dose of F.S.H. followed by diminishing daily doses might overcome this complication, but the results failed to confirm this and suggested that a constant daily dosage was more effective.

The most abnormal responses were observed when H.C.G. was given for the longest duration of time. These occurred in the two most sensitive patients in Experiment 1A (Figure 8, A5, B5). Since the two least sensitive patients failed to show similar responses it appears that this effect of H.C.G. is dependant on an adequate dosage of F.S.H. Indeed none of the patients showed any response to 24,000 i.u. H.C.G. when given without F.S.H. in the control period.

A rather similar abnormal response occurred in Experiment 1 when the most sensitive patient was given

the preparation of F.S.H. which was most heavily contaminated with luteinizing hormone (Figure 4,A4). Clearly the interaction between these hormones requires further study.

The clinical responses to the trial have been interesting. Uterine bleeding occurred in only 18 of the 47 months during which follicle stimulating hormone was given. These responses are summarised in Table X11. Menstruation was described by the patient as normal in 12 and as scanty in six of these. It is surprising that large responses with a normal pattern in the excretion of both oestriol and pregnanediol like those shown by Patient A in Figure 9, A8 and by Patient E in Figure 14, E12, were not followed by menstruation. In contrast bleeding occurred in other months when there was little or no increase in steroid excretion as shown in Figure 9, B7, B8, B9, C7, C9 and Figure 14, B10, C11, and E13. In some of these instances it appears that the bleeding occurred in the month after, rather than in the month of, a good rise in steroid excretion (Figure 4, B3 and 14, E13). Uterine bleeding therefore appears to be poorly related to the steroid response.

Comparison with other published work:

Gemzell et al (1958) using a crude preparation of human pituitary F.S.H. succeeded in inducing ovulation and ovarian enlargement in women with secondary amenorrhoea and with the Stein-Leventhal Syndrome. The changes observed were accompanied by marked increases in the levels of urinary oestrogens and pregnanediol with changes in endometrium from an atrophic to a secretory state in most cases. It was found that some of the patients treated produced oestrogen and pregnanediol changes when treated with F.S.H. alone, but the greatest changes occurred when F.S.H. was followed by H.C.G. The preparation of F.S.H. used by Gemzell and co-workers was contaminated with L.H. so this probably explains the effect of the preparation on the production of steroid by the ovaries of some of the women who were treated.

In this work the effect of F.S.H. alone on production of steroids was never studied on its own but when a patient with Simmonds's disease was treated with F.S.H. a steady rise in oestriol excretion occurred

TABLE XII

Uterine Bleeding During the Three Experiments

Experiment No.	Patient	Month	Day of Cycle	Duration in Days	Amount of Bleeding
I	A	3	24	7	Normal
	B	3	25	7	Normal
IA	A	5	24	4	Normal
	B	5	25	4	Normal
II	B	6	24	5	Scanty
	B	7	10	2	Scanty
	B	8	15	1	Scanty
	B	9	25	5	Normal
	C	7	24	3	Scanty
	C	8	21	6	Normal
	C	9	19	3	Scanty
	D	6	24	6	Normal
	D	8	(pregnant)		
III	A	10	19	6	Normal
	B	10	26	5	Normal
	B	12	25	6	Normal
	C	10	23	5	Normal
	C	11	25	2	Scanty
	E	13	10	5	Normal

but in the patients with secondary amenorrhoea no increase had taken place in either oestriol or pregnanediol by the fifth day of treatment with F.S.H. In Experiment 2 large doses of F.S.H. were given at the beginning of some months and the respective doses were reduced by constant factors, so that in some cases comparatively little F.S.H. was given before H.C.G., nevertheless no changes had occurred in steroids up to the fifth day of treatment with F.S.H. The responses of a women with Simmonds' and a women with the Stein-Leventhal Syndrome will be discussed in separate sections.

The question of how long after treatment with F.S.H. would H.C.G. be effective in inducing ovarian changes was investigated by Johannisson, Gemzell & Diczfalusy (1961). They administered a large dose of F.S.H. in a single injection to 5 amenorrhoeic women (2 had primary and 3 had secondary amenorrhoea) and found a continuous rise in the oestrogen excretion which reached a peak value between the sixth and seventh days after F.S.H. This they interpreted as being due either to oestrogen production over a long period or to the slow disappearance of F.S.H. activity from the body so that a continuous stimulation was maintained for a week or so. When H.C.G. was administered within 48hr. following the injection of F.S.H., the combined treatment caused a rapid rise in oestrogen and pregnanediol production and since the extra boost to the production of steroid maintained the elevated steroid levels for about fourteen days, they interpreted the oestrogen production as being due to oestrogen formation by the corpus luteum. When the interval betwen treatment with F.S.H. and H.C.G. was extended to 96hr. the injection of H.C.G. did not bring about any change in steroids which indicated that the follicles were no longer susceptible to the luteinizing action of H.C.G. These observations support the present findings that the responses by the ovary are not only dependent on the total amount of gonadotrophin administered but are also dependent to the relative proportions of L.H. to F.S.H. used and the time interval between treatment. This is clearly shown in Patient D, Experiment 2, Month 1 when an enormous response to gonadotrophin occurred in the levels of both oestriol and pregnanediol while slight alteration of the total dose and the rate of fall off of F.S.H. resulted in no responses, while with an

intermediate total dose in month 3 the patient showed a response similar to month 1. In each of these three months the only factors that changed were the total dose of F.S.H. and the rates of fall off of F.S.H. which suggests that the balance of circulating hormone in women is important in the induction of ovulation, Any defects in this hormone balance would be expected to lead to anovulation and the development of follicles that fail to rupture.

The outstanding differences between the responses obtained in this work and those of Gemzell and co-workers is in the degree to which patients responded. Slightly different systems to collect and pool urine specimens were adopted but nevertheless some of the patients of Gemzell and co-workers frequently produced oestriol levels above 1mg./24hr. and pregnanediol levels to 20mg./24hr., the only patient who had a response comparable with any of Gemzell's patients was Patient D in Experiment 2. From the work of Gemzell it is not possible to ascertain how long the levels of steroids remained elevated or if the values published were maximum values since only a few specimens of urine were collected during each month of treatment. Nevertheless there was a high incidence of multiple pregnancies in Gemzell's series of patients which suggested that the abnormally high responses during treatment were the result of multiple ovulation (Gemzell 1962). This is clearly undesirable.

Gemzell (1960) has shown that no antihormone production resulted from repeated treatments with human gonadotrophins and identical steroid responses accompanied by menstruation were obtained in a women with secondary amenorrhoea who had been treated seven times during the course of one year. In the present work also the patients retained their responsiveness to gonadotrophins after several months of treatment and ultimately all became pregnant. The same treatment was not repeated in any two consecutive months in any patients due to the statistical design of the experiments.

The effects of increased doses of F.S.H. used with constant high doses of H.C.G (6,000 i.u./day for 5 days) has been investigated by Rosemberg et al (1962) and their results support the findings reported here. They found increased excretion of steroids with increased doses of

F.S.H. and sometimes a secondary peak in the excretion of oestriol occurred.

Gemzell et al (1958) reported some patients who failed to respond to the dosages of gonadotrophins usually used, these women were found to be lacking in germinative tissue on examination of the ovaries. This led Gemzell to adopt a standard test dose of F.S.H. and H.C.G; patients were considered unsuitable for further treatment if they failed to respond. It is interesting to note that of the four patients in the first trial Patient D would probably have been rejected under this scheme, but after increased dosage of F.S.H. in Experiment 2 she became pregnant.

Other methods of assessing ovarian function have included vaginal cytology. Johannisson et al (1961) compared this method with the chemical method for oestrogens and pregnanediol but since the correlation was not always good it was decided to rely on the determination of steroids in this work.

Pregnancies after treatment with gonadotrophins:

It is interesting to note that pregnancies occurred in patients A, B and C following the type of treatment with gonadotrophin that was suggested by the results of the preceeding experiments. In addition the treatment with dexamethasone in patient C was probably also effective. The high resting level of pregnanediol in this patient was reduced by treatment with dexamethasone which indicated that it was of adrenal origin. It is possible that at the same time as treatment with gonadotrophin some adrenal steroid was suppressed which had previously inhibited ovulation. Diczfalusy (1962) showed that certain androgens, and Gemzell (1960) showed that progesterone inhibited the effect of exogenous gonadotrophin.

Brown (1956) has shown that when fertilisation takes place the level of oestriol remains elevated after the initial peak at ovulation. The four patients in this work who became pregnant provided an opportunity to study the excretion of hormones from the time of ovulation and throughout pregnancy.

Excretion of H.C.G. during pregnancy.

There are as yet few reports on the immunological estimation of H.C.G. during pregnancy. Wide (1962) has found that the values are usually higher than those

obtained by bioassay during the last two trimesters of pregnancy but unpublished observations from this laboratory suggest that the results obtained have been more in accord with bioassay.

Between weeks 9 - 10 the level of H.C.G. in Patient D reached 500,000 i.u./24hr. It dropped to an average of 20,000 i.u./24hr. for the rest of pregnancy.

Excretion of H.C.G. in patients A and B followed the normal pattern but increased slightly during steroid therapy. Smith & Smith (1948) suggested that a reciprocal relationship exists between the level of H.C.G. and oestrogen in urine and blood during pregnancy. They advanced the hypothesis that H.C.G. is utilised by the placenta to promote the synthesis of oestrogen. Work by Loraine (1958) was unable to support such a hypothesis but the importance of H.C.G. for the utilization of citrate by placental homogenates has been shown by Treon and Gordon (1958) and H.C.G. has been found necessary for the conversion of oestradiol to oestriol, 2 methoxy-oestrone and 16-epioestriol by perfused placentae (Treon 1959, 1961).

The excretion of H.C.G. in Patient C was very low in contrast to the other three patients, the failure to produce H.C.G. has been explained by Diczfalusy (1961) as being due to failure of the trophoblast to implant into the uterine bed. It was not known what effect dexamethasone may have exerted at the early stages of pregnancy, so that the dose was decreased in stepwise manner. The finding of a low level of H.C.G. in early pregnancy is claimed by Zondek and Goldberg (1957) to be a better indication of any early abortion than is given by the measurement of steroids and the present results support this view.

The excretion of oestriol during pregnancy.

The excretion of oestriol during the pregnancies of Patients A,B and D makes an interesting comparison. In Patient A the excretion of oestriol was within the normal range until week 30 when there was a steady fall from 14.9mg./24hr. to 6.5mg./24hr. The excretion increased again during treatment with steroids to within the normal range.

The excretion of oestriol during the pregnancy of Patient B was low and on week 31 it fell to 3.1mg/24hr. During treatment with steroids it returned to its former level of 5.0mg./24hr.

In contrast the level of oestriol in Patient D was abnormally high soon after treatment with gonadotrophin and by week 16 it had reached 4.3mg./24hr. It remained in the upper half of the normal range as is usual in twin pregnancies.

The excretion of oestriol in Patient C was within the normal range until about the 45th day when it gradually fell up to the time of operation.

Excretion of Pregnanediol during Pregnancy.

All of the patients showed a high excretion of pregnanediol in the early stages of pregnancy with a characteristic fall in excretion when the excretion of H.C.G. rose. The excretion of pregnanediol then remained within the normal range in Patient A until the 29th week when the value fell from 37.0mg/24hr. to 15.2mg./24hr. by the 31st week. During treatment with steroids it rose together with oestriol and at the end of pregnancy had approached the normal mean. In contrast the pregnanediol level in Patient B remained at about the lower limit of the normal range until the 29th week when it fell from 20.0mg./24hr. to 10mg./24hr. on week 31. During treatment with steroids only a slight increase occurred in the pregnanediol level. The excretion of pregnanediol in Patient C was normal until about day 50 after which it gradually decreased up to the time of operation. The initial rise in pregnanediol in Patient D was grossly abnormal, reaching 84mg./24hr. which is not usually encountered even at full term. The progesterone level in blood was 10.2µg./100ml. of plasma at this time, this level was between three to five times as high as normal values for this stage of pregnancy which suggests that multiple ovulation had occurred. The excretion of pregnanediol however fell gradually and remained about the normal mean value until delivery. Menstruation has not occurred since in any of the patients that became pregnant during treatment with gonadotrophins.

The fall in the excretion of steroids during the pregnancies of Patients A and B was more than could be accounted for by daily fluctuation and was probably the result of failing function on the part of the placentae. The level of urinary oestriol is considered by most workers to reflect the viable state of the foetus (Breitner, 1954; Zondek & Goldberg, 1957; Zondek & Pfeiffer, 1959; Ten Berge 1959, 1960, Kellar, Matthew, MacKay, Brown & Roy, 1959; and Furuhjelm, 1962). The work of Cassmer (1959) has shown that the separation of placenta from foetus in vivo led to a parallel drop in all oestrogens but in situ perfusion of the placenta restored the oestrogens within one hour.

On the other hand the level of pregnanediol in the urine is thought to be more dependent on the presence of the placenta rather than the viable state of the foetus. The pregnanediol level can remain elevated in cases of intra-uterine foetal death (Appleby & Norymberski, 1957; Diczfalusy, 1961) and in two cases of abdominal pregnancy Allen (1953) showed that after removal of a living child with the placenta left in situ, the pregnanediol level remained elevated for several weeks. Cassmer (1959) showed that after induced foetal death the pregnanediol levels remained elevated until removal of the placenta.

The fall in both oestriol and pregnanediol levels clearly indicated placental failure and steroid therapy was begun using the biologically active progestagen 17-α hydroxy progesterone caproate (Zarrow, Neher, Lazo-Wasem & Salhanick, 1957; Reifenstein, 1957). This compound is more active biologically than progesterone and has a prolonged action due to slow absorption from the injection site. In addition the ester is unchanged whereas progesterone itself is partly metabolised (Davis & Wied, 1955; Plotz & Davis, 1960).

The source of oestrogen in the treatment of Patient A was principally norethisterone with smaller quantities of oestradiol monobenzoate and ethinyl oestradiol. The last two steroids were also given to Patient B.

The method of Klopper et al (1957) was suitable for use during steroid therapy since 17-α hydroxy progesterone caproate is not excreted in the urine as pregnanediol or pregnanetriol (Bongiovanni, Eberlein & Cara, 1957; Brooks, 1960 and Plotz & Davis. 1960).

The work of Brown & Blair (1960) and Breuer, Dardenne & Nocke (1960) suggests that neither ethinyl oestradiol or norethisterone contribute to an increase in urinary oestriol as measured by the method of Brown (1955) or can be modified by any chemical stage in the method so as to contribute to the "oestriol" fraction. It is probable that 17-α hydroxy progesterone caproate is metabolised to oestrogen by the placenta but a striking difference was seen between the oestriol excretion patterns of Patient A and Patient B who was treated with much greater quantities of 17-α hydroxy progesterone caproate.

The fall in the excretion of steroids during the later stage of pregnancy may be due to a variety of causes. It may be due to increased metabolism of steroid precursors to other compounds or a change in the biological "half-life" of progesterone. The work of Pearlman (1957) suggests that the turn over rate of progesterone was the same in both pregnant and non-pregnant women but no study has been undertaken in cases of placental failure. The increased steroid levels during therapy may be due to an improved vascular supply to the placenta as well as to the fact that beneficial results can be obtained in some cases by confining the patient to bed. The results in Patient B show the value of frequent estimations during pregnancy. The absolute amount of oestriol excreted does not give an accurate assessment of placental function but the trend in the excretion of oestriol is important.

Discussion to Section III

Treatment of a woman with Simmond's Disease using human gonadotrophins.

The response to exogenous gonadotrophin by the patient with Simmonds' disease differed from that of the other patients studied. She produced very large amounts of oestriol but very little pregnanediol while the other patients were able to produce both steroids. When the dose of H.C.G. was given together with the last injections of F.S.H. the level of pregnanediol never rose above 3.0mg./24hr. although on two occasions the oestriol level rose to over 700µg./24hr.

The addition of other hormones did not appear to be successful. Menstruation occurred in some months but it was thought to be due to "withdrawal bleeding" after the fall in oestrogen secretion. It was found that the greatest response in oestriol occurred when H.C.G. was given together with F.S.H. and that intermediate responses were obtained when H.C.G. was given partly with and partly after F.S.H. In month 1 the level of oestriol increased after treatment with with F.S.H. alone. This has not been found to occur in the women with secondary amenorrhoea but occurred in the patients with the Stein-Leventhal Syndrome.

It is known that progesterone is a metabolite on the biosynthetic pathway leading to the formation of oestrogen in the human ovary (Smith & Ryan 1961, Ryan & Smith, 1961 a,b) and it has also been found in the follicular fluid from ovaries of normal women and women with the Stein-Leventhal syndrome (Short & London 1961; Short, 1962). It is probable that in this patient the pregnanediol excreted is derived from the progesterone formed in the metabolic pathway leading to oestrogen formation rather than from progesterone formed by luteal tissue resulting from ovulation. Similar types of cycles have been described by Brown & Matthew (1962) in women with cystic glandular hyperplasia. In these women the oestriol excretion rose to a high assymmetrical peak at mid-cycle and was followed by menstruation when the oestrogen level fell. No change occurred in pregnanediol and the cycles were considered to be anovulatory.

Gemzell (1960) found that a large increase in oestriol excretion occurred but little pregnanediol was excreted when a hypopituitary dwarf was treated with HP-F.S.H. Ovarian enlargement and ovulation has been reported by Gemzell (1961) after HP-F.S.H. followed by H.C.G. in another hypopituitary dwarf with primary amenorrhoea and hypogonadism, but again the greatest change occurred in the excretion of oestriol. The oestriol level rose to over 400µg./24hr. but the pregnanediol remained at less than 1mg./24hr.

The differences in the type of responses obtained by women with impaired pituitary function suggests that longer treatment with H.C.G. is necessary before ovulation can be induced and that the relative doses of F.S.H. and LH used in this work were incorrect.

The effect of treatment with gonadotrophin on the excretion of steroids by women with the Stein-Levethal Syndrome.

In this study the design of Netter et al (1961) was used, whereby adrenocortical activity was suppressed by the administration of dexamethasone and simultaneously ovarian activity was stimulated by treatment with gonadotrophins. Thus any defects in the production of ovarian steroids should be shown up.

In all patients the excretion of oestriol increased after treatment with dexamethasone. It has also been shown that this is preceeded by an increase in the excretion of F.S.H. (Butt, Crooke, Cunningham & Palmer 1963). The increase in oestriol is also evident from the results of Netter et al (1961). After treatment with F.S.H. and F.S.H. followed by H.C.G. the excretion of oestriol increased again. Netter et al (1961) also noted increased excretion of oestriol following H.C.G. alone but little change in oestrone and oestradiol. Thus it was postulated that there was a defect in the metabolism of oestrogens which lead to an exaggerated excretion of 16-OH metabolites such as oestriol and 16-hydroxyoestrone.

The excretion of pregnanediol was suppressed by dexamethasone presumably because some of it arose from the adrenal cortex.

As in the experiments on patients with secondary amenorrhoea it is clear that a normal ovulatory pattern in the excretion of oestriol and pregnanediol is only obtained with certain combinations of F.S.H. and H.C.G. When the time interval between injections of F.S.H. was reduced to 4 days a very large increase in oestriol occurred accompanied by very high levels of pregnanediol. When the interval between injections of F.S.H. was 12 days a normal pattern was obtained. The dosage was also important in the group of women with secondary amenorrhoea and some only responded to very high doses of F.S.H. In this series, however, only one patient (N) failed to respond to a total dose of 4000mg. equivalents F.S.H. and some showed enormous increases in oestriol. Thus it seems that in general these patients are more sensitive to exogenous gonadotrophin.

The effect of gonadotrophin on the excretion of Dehydroepiandrosterone.

Dexamethasone completely suppressed the excretion of D.H.A. in the first 3 patients investigated (Patients G, H and I) and furthermore D.H.A. was not detected after simultaneous treatment with gonadotrophin. Similar results have been reported by Netter et al(1961). However in the other patients (J and K) it was found that when injections of F.S.H. were given at intervals of 4 days the excretion of D.H.A. did increase which suggests that some D.H.A. is of ovarian origin since there is no evidence that the preparation of F.S.H. had stimulated the adrenal cortex and in view of its method of preparation would be unlikely to be contaminated by A.C.T.H. Similar results have been reported by Mahesh & Greenblatt (1961), and Mahesh, Greenblatt, Aydar & Roy (1962) following treatment with β-methasone and human pituitary F.S.H. D.H.A. was also found in the fluid obtained from follicular cysts (Mahesh et al 1962; Mahesh & Greenblatt, 1962).

It is interesting that in the present studies both D.H.A. and oestriol were detected in the urine, If the blockage in 3β-ol dehydrogenase was complete (Mahesh & Greenblatt, 1961), no formation of oestriol would be expected since the principal metabolic pathway from pregnenolone. — 17α hydroxy pregnenolone - D.H.A. - oestrogen would be blocked at D.H.A. The oestriol may therefore be formed from precursors on a different metabolic pathway which may be pregnenolone - progesterone - 17α hydroxy progesterone - androstenedione - 19-hydroxy androstenedione - oestrogen. These two metabolic pathways have been established by the work of Ryan & Smith (1961 a,b) and Smith & Ryan (1961). Furthermore Mahesh & Greenblatt (1962) showed that slices of polycystic ovaries that contained large quantities of D.H.A. formed oestrogen from androstenedione as precursor but failed to form androstenedione when incubated with D.H.A.

The effect of gonadotrophin on the excretion of Fractions II and III.

Dexamethasone reduced the levels of both fractions II and III and little change occurred in fraction III during simultaneous treatment with gonadotrophin. The level of fraction III indicates the excretion of 11-oxygenated corticosteroids and thus demonstrated that adrenocortical steroid production remained at a fairly constant level during treatment with gonadotrophins. On the other hand fraction II which reflects the excretion of pregnanetriol, increased after treatment with gonadotrophins and presumably was of ovarian origin. Pregnanetriol has also been found in increased quantities after treatment by Mahesh & Greenblatt (1961). There is good evidence that pregnanetriol may be of ovarian origin from studies during the menstrual cycle of normal women (Pickett, Kyriakides, Stern & Sommerville, 1959; Fotherby, 1960; Pickett & Kellie, 1962; Pickett & Sommerville, 1962 and Fotherby, 1962). The ovarian precursor of pregnanetriol is thought to be 17α-hydroxy progesterone which has been found in human ovarian tissue (Zander, 1958) and in follicular fluid (Short, 1962). It has also been found in the follicular fluid of patients studied in this work, when increased quantities were found coincident with increased amounts of progesterone and elevated levels of urinary pregnanediol. It has also been demonstrated that 17α hydroxy progesterone and androstenendione can be formed from progesterone in both human and bovine ovaries (Salmon, Vande Wiele & Lieberman, 1956; Wiest, Zander, Holmstrom, 1959; Sweat, Berliner, Bryson, Nabors, Haskell & Holmstrom, 1960, Kase, Forchielli & Dorfman, 1961; and Axelrod & Goldzieher, 1962).

The effect of treatment with gonadotrophin on the excretion of 17-oxogenic and 17-oxosteroids.

It is well established that the ovary is capable of producing steroids excreted as 17-oxosteroids (Borell, 1954 and Personen & Timonen & Mikkonen 1959) and that increased quantities of 17-oxosteroids and 17-hydroxy corticosteroids are excreted after stimulation of human ovaries by treatment with gonadotrophin (Keetal et al, 1957; Gemzell et al, 1958; Mahesh & Greenblatt, 1961). The failure to achieve complete suppression of the level of 17-oxosteroids by a standard dose of dexamethasone is used by Netter et al (1961) as a diagnostic test to separate patients with menstrual irregularities into those who have elevated production of androgen from the ovary and those who have not.

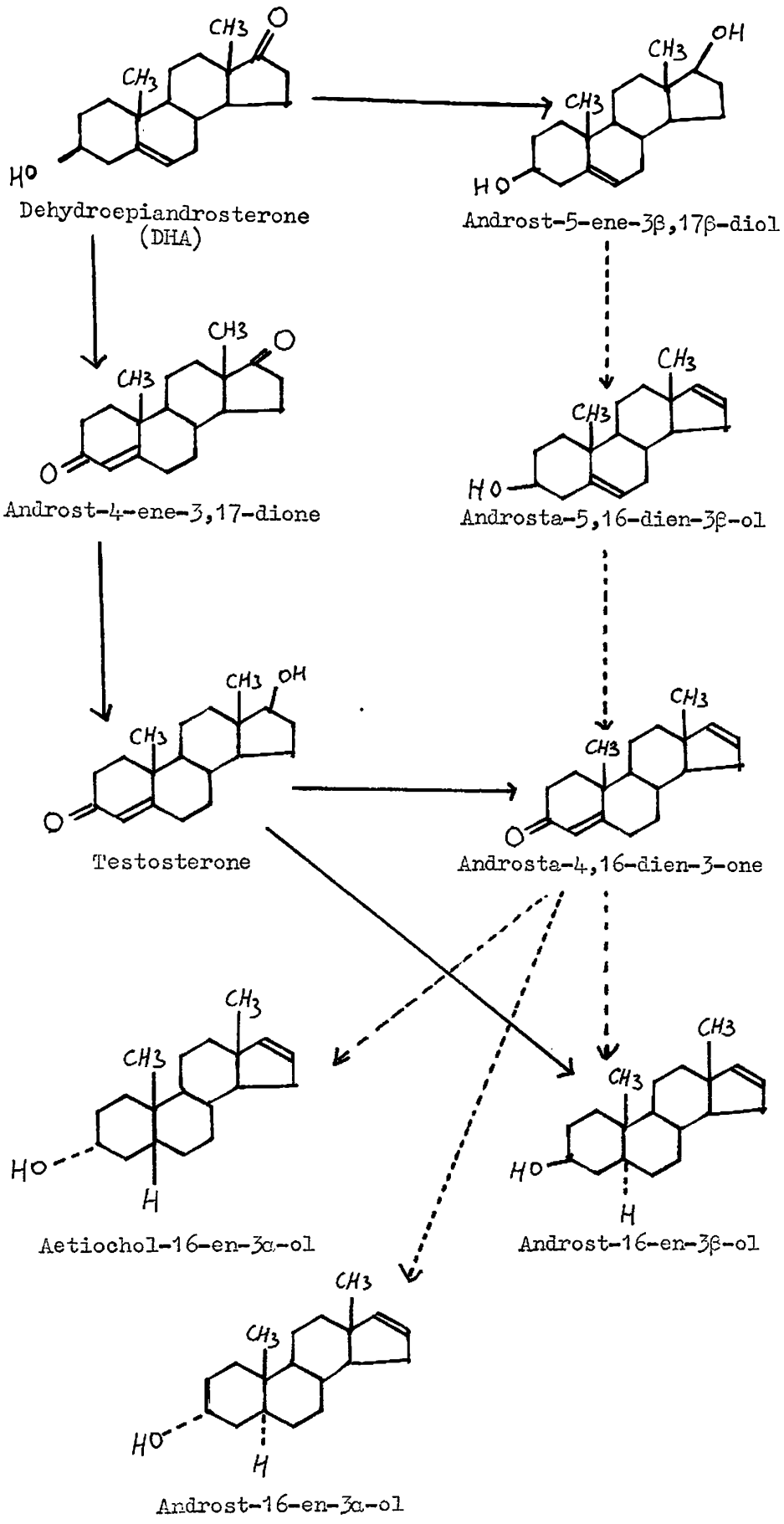
In this work it was found that dexamethasone failed to suppress the level of 17-oxosteroids in one patient (G) but suppressed the levels of 17-oxosteroids to different degrees in patients H and I. Treatment with gonadotrophin increased the level of 17-oxosteroids in both patients G and H.

In the patients not treated with dexamethasone increased levels of 17-oxosteroids and 17-oxogenic steroids occurred in four of the five patients after treatment with gonadotrophin. To explain similar findings Gemzell et al (1958) suggested that the results may be due to (1) contamination of the gonadotrophin with A.C.T.H but in the preparations of Gemzell and in these preparations contamination by A.C.T.H. can be precluded by the technique used to prepare the gonadotrophins. (2) It may be possible that the gonadotrophins may exert a direct action on the adrenal cortex - it is not possible to verify this until the sequence of amino acids in the gonadotrophin molecules is elucidated and tested for A.C.T.H. activity. (3) The most likely source of the increased 17-oxogenic and 17-oxosteroids is from the increased production of ovarian steroids. It has been amply demonstrated that the ovary is capable of producing metabolites of 17 α hydroxy progesterone and progesterone. Furthermore in this work increased quantities of 17-α hydroxy progesterone has been shown to occur in follicular fluid after treatment with gonadotrophin. Thus it appears that the ovary is the site of production of the increased levels of 17-oxogenic and 17-oxosteroids.

The effect of treatment with gonadotrophin on the excretion of Androstenol.

Dexamethasone decreased the levels of androstenol which indicated that the adrenal cortex was the principal source of urinary androstenol. The use of A.C.T.H further established this. Nevertheless even in the presence of dexamethasone increased levels occurred after treatment with gonadotrophins. When the ovaries of patients J, K, L and M were subjected to increased stimulation by reducing the time intervals between injections, the levels of androstenol increased. This suggests that the ovary too can form androstenol, since no change occurred in the excretion of fraction III showing that adrenocortical activity remained relatively constant. Brookshank (1962) found that when normal men and women were treated with A.C.T.H, increased levels of androstenol resulted and in men increased levels were also found after treatment with H.C.G. (1,000 i.u./day for 3 days) which suggests that androstenol was of gonadal as well as adrenocortical origin. The rise in the excretion of androstenol in both sexes after puberty and the significant decrease in androstenol in women over 45 years suggests that a connection exists between gonadal function and androstenol excretion. The exact biosynthetic pathway leading to the formation of androstenol is speculative at the moment.

A hypothetical pathway has been proposed by Brooksbank (1962). This is shown diagramatically. This was based on the finding of Gower & Haslewood (1961) that the testis of rats can form androstenol in vitro from acetate. The conversion of testosterone in vitro to the closely related androsta - 4:16 dien-3-one by human liver (Stylianou, Forchielli, Tummillo & Dorfman, 1961, b) and to the latter and androst - 16-en - 3β ol by rat testis has been demonstrated (Stylianou et al, 1961,a). However, the marked enhancement of androstenol excretion by A.C.T.H. indicates that it does not arise only from testosterone (via androsta - 4;16 - dien - 3 - one) but can be formed in vivo from adrenal steroids. In view of the importance of D.H.A as a product of the adrenal (Vande Wiele & Lieberman, 1960) a possible origin of androstenol would seem to be from D.H.A. via androst- 5 - ene - 3β, 17 β diol



Possible metabolic interrelations between androgens and Δ^{16}-steroids

(Ungar, Miller & Dorfman, 1954), androsta- 5, 16 - dien 3B - ol and androsta - 4, 16 - dien 3 - one. These reactions are also shown.

It has been demonstrated that testosterone can be formed by human ovarian tissue (Sandor & Lanthier, 1960; Kase et al, 1961, and Leon, Castro & Dorfman, 1962) and testosterone can be converted into oestrogen by human ovarian tissue (Baggett, Engel, Savard & Dorfman, 1956; Wotiz, Davis, Lemon & Gut, 1956, West, Damast, Sarro & Pearson, 1956, Ryan 1959, Ryan & Smith 1961a). The findings in this work indicate that the human ovary can also form androstenol. The findings of Ryan,(1959) on the aromatization of steroids by human placental homogenates showed that 16-α hydroxy testosterone was an intermediate in the conversion of Δ 5 androstene 3B, 16α, 17B triol and can be formed from D.H.A. by perfusion through intact rat liver (Ungar et al, 1954). This suggests that androstenol may play a key role in the production of androgen as has been proposed by Dorfman (1961). 16-α hydroxytestosterone has been found by Stylianou et al (1961 b) when $4C^{14}$ testosterone was incubated with human liver slices, so that this compound, 16-α hydroxy testosterone, may be of more importance in the metabolism of androgens and oestrogens than androsta- 4 : 16 dien - 3 one as proposed by Brooksbank (1962).

The steroids present in follicular fluid.

The presence of relatively large quantities of androstenedione in the follicular fluid of patients H and I was similar to the findings of Short & London (1961), Short (1962a). Both patients excreted little pregnanediol at the time at which the follicular fluid was removed and the oestriol level was either low or had decreased. In keeping with the work of Short & London (1961) and Short (1962a), oestradiol-17 β was not detected.

A marked difference was noted between patients H and I and patients J and K. The follicular fluid was removed from the latter when the excretion of urinary oestriol and pregnanediol was elevated and it contained relatively large quantities of progesterone, 17-α hydroxy progesterone and oestradiol-17β with decreased levels of androstenedione. A small quantity of oestrone and 20α hydroxy pregn - 4 - en - 3 - one was detected in the follicular fluid from patient K. Short (1962b) has found the latter steroid in high concentration in luteal tissue of the mare but could detect none in the follicular fluid of the same animal. Oestrone and oestradiol-17 β have been detected in extracts of corpora lutea by Zander, Brendle, von Munstermann, Diczfalusy, Martinsen & Tillinger (1959). The finding of progesterone and 17α hydroxy progesterone in these latter patients demonstrated the effect of the gonadotrophic hormones on the constituents found in the follicular fluid. Warren & Salhanick (1961) claimed that the defective enzyme system in the ovaries of women with the Stein-Leventhal Syndrome lay in an excessive activity of the enzyme 17α hydroxylase rather than an enzyme blockage. This work clearly shows that the relative amounts of steroids found in follicular fluid was dependant upon the predominant hormone acting on the ovary immediately before the follicular fluid was removed. If L.H. had been the active hormone then increased progesterone and 17α hydroxy progesterone would be expected to be present due to the luteinizing action of L.H.

It is now known that when a mature follicle ruptures either by a mechanical means or by the action of L.H. that the biosynthetic pathway leading to the formation of oestrogen is broken in some way and that

the metabolic pathway is arrested at ~~at~~ - progesterone so accounting for the increased level of pregnanediol that occurs after ovulation. A change in endocrine cell type occurs at ovulation also (Harrison, 1946). It is known that 2 types of endocrine cells exist in the normal ovary, these are the theca interna which contain all the enzyme systems necessary for the conversion of progesterone to oestradiol 17 B and the granulosa cells which lack these enzymes and produce mainly progesterone (Short, 1962 a, Huang & Pearlman, 1962).

The changes found in the constituents of the follicular fluid pf patients J and K. The cyst fluid of Patient L had both D.H.A. and androstenedione present in the same follicles with small quantities of 17α hydroxy progesterone also. The occurrence of both androstenedione and D.H.A, together in the same follicular fluid has been reported by Short (1962a). Patient N had a small quantity of 17-α hydroxy progesterone but considerably more androstenedione. These findings indicated that the changes that occurred after treatment with gonadotrophin were of a transient nature and that the steroids found in the cyst fluid of untreated women with the Stein-Leventhal Syndrome re-appear when treatment has been discontinued. It thus appears that under the continual influence of gonadotrophin, the enzyme defect in the biosynthesis of oestrogens may be corrected at least temporarily. It is not possible to decide if the follicles present after gonadotrophin treatment had been already present before treatment or whether they are newly formed follicles. On regression these follicles had the cyst fluid constituents of untreated women.

Several theories have been advanced to explain the occurrence of polycystic ovaries in the Stein-Leventhal Syndrome. Originally hypergonadotrophinism was cited as the basis of the polycystic ovary (Stein & Leventhal 1935) but it was found by McArthur, Ingersoll & Worcester (1958) that the excretion of L.H. in urine was not always elevated in these patients. Taymor & Barnard (1962) showed that L.H. was increased in about 50% of a small group of women with the Stein-Leventhal Syndrome, ~~but with cystic ovaries.~~ Considerable fluctuation occurred in the excretion of L.H. in all of the patients studied. The response of some patients

to corticoid therapy has implicated the adrenal cortex into consideration (Greenblatt 1953, 1958, Jones, Howard & Langford, 1953), and it has been proposed by Gallagher, Kappas, Hellman, Lipsett, Pearson & West (1958) that the abnormality may involve dysfunction of adrenal gland, pituitary gland and ovary. Mahesh & Greenblatt (1961) suggest that since the ovaries are so responsive to exogenous gonadotrophin that hundreds of follicles receive inadequate stimulation by endogenous hormones so producing a myriad of micro follicular cysts with general enlargement of the ovary. The possibility also exists that the syndrome may be related to the sex chromatin pattern since the report by Vague, Picard, Vitry, Galinier, Favier & Miller (1956) who found a male sex chromatin pattern in the buccal smear of one of the group of four patients with the Stein-Leventhal Syndrome.

SUMMARY

1. In this research work a short rapid method for the estimation of oestriol in urine was developed.

2. This method was compared to a longer published method and found to be suitable for the estimation of small quantities of oestriol and for following the excretion of oestriol during pregnancy.

3. This method was discussed with reference to accuracy, specificity, sensitivity and precision.

The effects of different preparations of human urinary and pituitary gonadotrophins on the excretion of oestriol and pregnanediol by a group of women with secondary amenorrhoea.

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4. Each period of treatment with gonadotrophins was designed in a statistical manner and the results were analysed by comparison to the changes in the excretion of oestriol and pregnanediol during the menstrual cycle of normal women.

5. In Experiment 1. three different preparations of F.S.H. were used followed by a constant dose of H.C.G. given for four days.

6. The analysis of the results indicated that the best preparation appeared to be CP2.

7. In Experiment 1a. each patient was treated with a constant high dose of CP2, and H.C.G. (6,000 i.u/day) was given for four days with the last four days of F.S.H. and for the four days after it.

The effect of varying the total and daily dosage of F.S.H. was investigated. The dose of F.S.H, decreased by factors of 0.7, 0.8, 0.9 in three of the four months and in one month a constant dose of F.S.H. was used. H.C.G. (6,000 i.u.) was given 4 days after treatment with F.S.H.

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8. One patient had an abnormally big response during the first month of treatment, no response at a lower dose and a big response at an intermediate dose level of F.S.H.

9. The analysis of results indicated that the high dosage of F.S.H. appeared to be more effective than the low dosages.

10. The use of a constant dose of F.S.H. appeared to be more effective than when different daily dosages were used.

11. During this experiment one patient became pregnant and was replaced in the group by another patient.

In Experiment 3 the total dosage of F.S.H, the total dosage of H.C.G. and the timing of administration of the two hormones was investigated.

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12. From the analysis of this experiment it appeared that the most effective way to give H.C.G. was in a dosage of 18,000 i.u. (or higher) in a single injection after F.S.H.

13. The analysis also suggested that the higher dosages of F.S.H. were more effective.

14. The graphic responses of these three experiments were compared to the excretion patterns of oestriol and pregnanediol during the normal cycle, by a method of awarding scores to different characteristics of the induced cycles but this did not supply any more information than the statistical analysis.

Using the information gained in the experiments 1 - 3 each patient was treated further with what appeared to be the most effective treatment to induce ovulation.

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15. Three of the patients treated in this manner became pregnant, two gave birth to normal single children, one aborted and the woman who became pregnant during Experiment 2 gave birth to dissimilar twins.

16. The excretion of oestriol, pregnanediol and H.C.G. during the pregnancy of each patient was studied.

17. A comparison was made between the excretion of oestriol and pregnanediol by three of these patients and the excretion patterns of normal pregnant women.

The effect of F.S.H. and H.C.G. on the excretion of oestriol and pregnanediol by a woman with Simmonds' disease was studied.

18. The results of giving H.C.G. in different ways relative to F.S.H. showed that the patient could produce large quantities of oestriol but comparatively little pregnanediol.

19. The greatest change in the excretion of both oestriol and pregnanediol occurred when H.C.G. was given together with the last four days of F.S.H.

20. The effects of other trophin hormones, dexamethasone, and thyroxin did not appear to supplement the action of the gonadotrophins in inducing a pattern of steroid excretion which indicated ovulation.

21. The scheme of treatment that had been used successfully to induce ovulation in women with secondary amenorrhoea was not successful in this patient.

In a group of women with the Stein-Leventhal Syndrome the hormones controlling the excretion of steroids were investigated.

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22. Dexamethasone suppressed the excretion of D.H.A completely and caused a reduction in the levels of androstenol, pregnanediol, fraction II and III and both 17-oxo and 17-oxogenic steroids.

23. After administration of dexamethasone the excretion of oestriol increased.

24. In the presence of dexamethasone, when F.S.H. alone was given the level of oestriol increased but little change occurred in any of the other steroids.

25. After the combined treatment with F.S.H. and H.C.G. the levels of pregnanediol, oestriol, androstenol, fraction II and 17-oxosteroids increased but the level of D.H.A. remained suppressed.

26. In the absence of dexamethasone and using a scheme of treatment which had a four day interval between injections of F.S.H. followed two days later by H.C.G., the levels of oestriol, pregnanediol, D.H.A., androstenol, fraction II and both 17-oxo and 17-oxogenic steroids increased.

27. When comparatively little change had occurred in the levels of urinary steroids, the cyst fluid removed at the same time contained comparatively large quantities of androstenedione.

28. When the levels of oestriol and pregnanediol were elevated in urine the cyst fluid removed at operation contained progesterone, 17-α hydroxy progesterone and oestradiol-17β.

29. When the cyst fluid was removed from patients at longer intervals following treatment with gonadotrophins, it was found to contain the same steroids that are found in the cyst fluid of untreated women.

30. Androstenedione and D.H.A. were found in cyst fluid which was removed from individual cysts of one patient.

The findings in this work were discussed and compared to the findings of other workers.

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STEROID NOMENCLATURE.

Trivial Name:	Chemical Name:
Androsterone	3α-Hydroxy-5α-androstan-17-one
Aetiocholanalone	3α-Hydroxy-5β-androstan-17-one
Androstenol	Androst-16-en-3α-ol.
Androstenedione	Δ^4-Androstene-3, 17-dione
Dehydroepiandrosterone (D.H.A.)	Δ^5-Androstene-3β-ol-17-one
Pregnanediol	5β-Pregnane- 3α 20α-diol
Progesterone	Pregn-4-ene 3, 20-dione
17-Hydroxyprogesterone	17α-Hydroxy pregn-4-ene-3, 20- dione
Pregnanetriol	5β-Pregnane-3α, 17α, 20α-triol
Pregnenetriol	Δ^5-Pregnene-3β,17α, 20α-triol
Pregnenolone	Δ^5-Pregnen-3B-ol-20-one
17-Hydroxy pregnenolone	17α Hydroxy Δ^5-Pregnen-3B-ol-20-one
Oestriol	3,16α, 17β-Trihydroxyoestra-1,3,5 (10) triene
Oestrone	3-Hydroxyoestra-1,3,5, (10)-trien-17-one
Oestradiol	3,17β-Dihydroxyoestra-1,3,5 (10) triene
Testosterone	17β-Hydroxy-androst-4-en-3-one

ACKNOWLEDGEMENTS.

I extend my thanks to Dr. A.C. Crooke for supervising and for attending to the clinical aspects of this work; to Dr. W.R. Butt for his unfailing help and encouragement at all times; to Mr. R. Morris and all members of the staff of the department for performing so many estimations; to Dr. R.V. Short of Cambridge for performing the estimations on the steroids in cyst fluid and for many valuable discussions, and to Dr. F.J. Cunningham who helped in preparing this work. My thanks are also extended to the patients, without whose co-operation in attending for investigations and diligently collecting specimens of urine, this work would not have been possible. I am also grateful to the Birmingham Regional Board for a grant to carry out this work.