Changes in total and free concentrations of steroid hormones in the plasma of women throughout pregnancy: effects of medroxyprogesterone acetate in the first trimester

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ABSTRACT

The total (protein-bound plus free) and free concentrations of progesterone, oestradiol-17β and cortisol were measured serially throughout pregnancy in the plasma of two groups of women whose pregnancies went to term. Group A ($n = 53$) experienced an uneventful low-risk pregnancy with a spontaneous abortion rate of 8·6%. Women in group B ($n = 22$) were treated orally with medroxyprogesterone acetate (MPA; 80–120 mg daily) until 18 weeks gestation for threatened abortion within the first 6 weeks of pregnancy.

In both groups of women the proportion of each hormone circulating in the free or unbound form remained constant despite the overall increases which occurred in total circulating hormone concentrations as pregnancy progressed. The steroid hormonal profiles in the first half of pregnancy were similar in both groups of women. However, from weeks 20 to 40 total and free progesterone concentrations were significantly ($P < 0·05$ in each case) higher in group B compared with group A. Conversely, total and free oestradiol-17β concentrations were lower ($P < 0·005$ and $P < 0·01$ respectively) in group B. At this stage it is not known if these differences were attributable to the administration of MPA to women in group B or to altered placental steroidogenesis as a result of earlier uterine bleeding.

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INTRODUCTION

The role of steroid hormones in normal and abnormal human pregnancies has been studied extensively in terms of the total concentrations of circulating hormones (Eton & Short, 1960; Tulchinsky, Hobel, Yeager & Marshall, 1972; Allen & Lachelin, 1978), especially near term when patients are seen more frequently (Shaaban & Klopper, 1973; Hartikainen-Sorri, Kaupilla, Tuimala *et al.* 1981). However, most of the steroid hormone circulating in blood is bound to proteins. The biologically active component of a steroid hormone is considered to be only the small fraction which circulates in a free or non-protein-bound form (Hoffman, Forbes & Westphal, 1969; Ekins, 1982).

The possibility that free hormone concentrations change independently of total hormone concentrations has not been examined throughout pregnancy in women. Previous studies have measured concentrations of free and protein-bound progesterone in late pregnancy in only a limited number of samples (Yannone, Mueller & Osborn, 1969; Batra, Bengtsson, Grundsell & Sjoberg, 1976; Greenstein, Puig-Duran & Franklin, 1977), while progesterone, oestradiol-17β and cortisol have been measured near term and during labour (Willcox, Yovich, McColm & Phillips, 1985), and androgens and glucocorticoids, rather than female sex steroids, have been measured by Booth, Dixon, Gray *et al.* (1961), O'Connell & Welsh (1969) and Forest, Ances, Tapper & Migeon (1971).

In this study total concentrations of progesterone, oestradiol-17β and cortisol were measured by radioimmunoassay, and their respective free fractions were determined in undiluted plasma at 37 °C by a rate dialysis technique (Willcox, McColm, Arthur & Yovich, 1983). Partitioning of cortisol in maternal

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plasma was investigated together with that of progesterone since both steroids are bound by transcortin and the relative affinity of transcortin for these hormones changes as pregnancy progresses (Seal & Doe, 1967; Rosenthal, Slaunwhite & Sandberg, 1969). Plasma samples were obtained from women with a history of sub-fertility. The women experienced either an uneventful low-risk pregnancy or received progestagen support therapy from weeks 6 to 18 of gestation because of threatened abortion within the first 6 weeks.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical grade, and solvents were redistilled before use. Peroxides and other oxidizing agents were removed from diethyl ether (Vogel, 1948) before distillation. Amersham International (Sydney, Australia) supplied [1,2,6,7-^{3}H]progesterone (87 Ci/mmol), [1,2,6,7-^{3}H]cortisol (85 Ci/mmol) and [2,4,6,7-^{3}H]oestradiol-17β (95 Ci/mmol). New England Nuclear (Boston, MA, U.S.A) supplied [6α-1,2-^{3}H(N)]methyl-17α-hydroxyprogesterone acetate (60 Ci/mmol). The radioactive steroids were routinely repurified by thin layer chromatography on silica gel using solvent systems recommended by the suppliers. Medroxyprogesterone acetate (6-methyl-17α-hydroxyprogesterone acetate; MPA) was provided by Upjohn Co. (Kalamazoo, MI, U.S.A.) for analytical use. Dialysis tubing (flat width 43 mm and mean dry thickness 0·02 mm) was purchased from Union Carbide, Chicago, IL, U.S.A.

Subjects

Ninety-one pregnant women, ranging in age and parity from 27 to 42 years and from 0 to 2 respectively, were recruited into this study. Their pregnancies followed treatment for primary or secondary infertility (arising from a range of causes) of from 3- to 8-years duration. Treatment consisted of ovulation induction with clomiphene citrate, either alone or in conjunction with human menopausal gonadotrophin, and tubal reconstructive surgery where appropriate. Conception was achieved following coitus, by artifical insemination (both donor and husband) or by in-vitro fertilization. All data were adjusted so that day 14 of the menstrual cycle was nominated as the day of ovulation and corresponded to the day of oocyte recovery or the day after the luteinizing hormone (LH) surge was recognized (all patients had daily periovulatory serum sampling for the detection of the LH surge). The women were divided prospectively into two groups (with and without progestagen support therapy) and sub-divided retrospectively according to the outcome of pregnancy.

Group A ($n = 53$) comprised sub-fertile women who underwent an uneventful low-risk pregnancy of normal gestational length (37–41 weeks) and delivered healthy infants.

Group B ($n = 22$) comprised sub-fertile women who had threatened to abort (uterine bleeding accompanied by a closed cervix) up to and including week 6 of gestation. Progestagen support was administered in the form of MPA (Provera; Upjohn Pty Ltd, Sydney, Australia; 20 mg orally 4–6 times daily) until week 16 of gestation. From week 16 the dose was progressively reduced and treatment ceased by week 18. The women delivered healthy infants between weeks 37 and 41 of gestation.

Group A initially comprised 58 women, but five women aborted before week 28 of gestation and were excluded from analysis. Similarly, 33 women were recruited originally into group B, but 11 of the women aborted spontaneously by week 8, despite MPA therapy, and were excluded from analysis.

In accordance with the University of Western Australia Committee for Human Rights, which approved the study, the informed consent of each woman was obtained before the start of sampling.

Plasma analyses

Blood samples (10 ml, heparinized) were taken from the antecubital vein, centrifuged and the plasma fraction stored in aliquots at −20 °C until analysis. Plasma was thawed only once for determination of free steroid fractions.

Albumin and total protein concentrations were determined by bromocresol green and biuret assays respectively, using a Cobas Bio autoanalyser (Roche Diagnostics, Dee Why, NSW, Australia). The between-assay coefficients of variation for albumin and total protein measured in this way were 1 and 2% respectively.

Progesterone, oestradiol-17β, cortisol and MPA were measured by radioimmunoassay procedures similar to that described by Thorneycroft & Stone (1972) for progesterone. Antiserum raised in goats against an MPA-3-oxime–bovine serum albumin complex was supplied by Dr K. T. Kirton (Upjohn, Kalamazoo, MI, U.S.A) and has been shown to be specific for MPA and closely related synthetic molecules (Cornette, Kirton & Duncan, 1971). In our hands MPA did not cross-react at 0·001% level in the progesterone assay and, conversely, progesterone did not cross-react at the 0·001% level in the MPA assay. The progesterone antiserum used (42-B5) cross-reacted 25% with 5α-pregnane-3,20-dione, which is reported to comprise between 7 and 23% of the measured progesterone in maternal plasma during pregnancy (Parker, Everett, Quirk *et al.* 1979). This may have caused an

overestimate of the total progesterone concentrations measured in this study. Steroid concentrations were corrected for procedural loss. Petroleum ether (boiling range 60–80 °C) was used to extract progesterone and MPA from plasma before assay with efficiencies of 75·3 ± 3·8% (mean ± S.E.M., $n = 15$ assays) and 79·7 ± 5·2% ($n = 5$ assays) respectively. Diethyl ether was used to extract oestradiol-17β and cortisol from plasma before assay with efficiencies of 84·8 ± 6·7% ($n = 12$ assays) and 74·6 ± 4·4% ($n = 9$ assays) respectively. The steroid extraction efficiencies were similar for plasma samples obtained during conception cycles, after conception and throughout pregnancy. Preliminary studies using 30–40 plasma samples obtained during the conception cycle and throughout pregnancy indicated that further purification of the organic extracts of plasma before assay was unnecessary. Regression analysis of the values obtained for samples assayed directly or after thin layer chromatography on silica gel gave correlation coefficients of 0·978, 0·965, 0·951 and 0·940 for progesterone, oestradiol-17β, cortisol and MPA respectively. Solvent blanks were equivalent to < 15 fmol/tube and the limits of sensitivity of the assays (per tube) were 80 fmol for progesterone, MPA and cortisol and 30 fmol for oestradiol-17β. This corresponded to detection limits in plasma of 0·6 nmol/l for progesterone and MPA, 0·5 nmol/l for cortisol and 0·3 nmol/l for oestradiol-17β. The intra- and interassay coefficients of variation of the five steroid assays were all ⩽7 and ⩽14% respectively. The free fractions of progesterone, cortisol and oestradiol-17β were measured in duplicate by rate dialysis. The method has been validated for human plasma and yields values comparable to those obtained by centrifugal ultrafiltration (Willcox *et al.* 1983). The intra- and interassay coefficients of variation of all measurements of free steroid fractions varied between 2·6 and 8·8% except for the intra-assay coefficient of variation of oestradiol-17β which was 10·2%. The free concentration of each steroid was obtained from multiplication of its free fraction by the total plasma concentration, determined separately by radioimmunoassay.

Statistical analysis

Results are expressed as means ± S.E.M. (n), where n is the number of observations. Mean free steroid fractions within each group were compared at different times during pregnancy by Student's t-test which was also used for comparisons between groups. Groups A and B were compared for protein and hormone concentrations by analysis of co-variance with time of pregnancy as the co-variant (Snedecor & Cochran, 1980). All concentrations were converted to logarithms.

RESULTS

The women whose pregnancies went to term in groups A and B delivered vaginally ($n = 59$) or by Caesarian section ($n = 16$) between weeks 37 and 41 of gestation. The mean birth weights (males plus female) and placental weights for group A were 3405 ± 67 and 597 ± 18 g and for group B were 3242 ± 100 and 566 ± 12 g respectively. Analysis of variance showed that there were no significant differences in these parameters between groups or between sexes in each group.

Plasma concentrations of protein and albumin declined similarly in the two groups as pregnancy progressed. Seven plasma samples with protein contents < 50 g/l were excluded from analysis. In both groups of women total plasma progesterone (i.e. protein-bound plus free hormone) increased progressively after implantation up to 34–36 weeks. Thereafter, the mean concentration declined before the onset of labour (Fig. 1*a*), although a prepartum decline was not observed in all individuals. The prepartum decline was significant ($P < 0·01$) only for total progesterone of group A when weeks 36 and 40 of gestation were compared. The free fraction of progesterone in plasma averaged 2·80 ± 0·04% (191) and 2·89 ± 0·05% (64) for groups A and B respectively and did not vary significantly during pregnancy either within or between groups. Consequently, the free plasma concentration of progesterone reflected, at a lower level, changes in its total plasma concentration (Fig. 1*b*). However, whilst the rate of increase of total and free progesterone was similar in both groups of women, sub-fertile women who had received progestagen support (group B) consistently had higher plasma concentrations of both total ($P < 0·005$) and free progesterone ($P < 0·005$) than those women who experienced an uneventful pregnancy (group A).

The free fractions of oestradiol-17β were constant throughout pregnancy (1·41 ± 0·09% (186) and 1·52 ± 0·10% (66) for women in groups A and B respectively). However, the rate of increase in total and free hormone was resolved into two components, from weeks 5 to 19 and from weeks 20 to 40, reflecting approximately the progression from ovarian to placental synthesis (Fig. 2). The plasma concentrations of total and free oestradiol-17β increased similarly in groups A and B up to week 19, but from weeks 20 to 40 the women receiving progestagen support had consistently lower plasma concentrations of both total ($P < 0·005$) and free oestradiol-17β ($P < 0·01$). Total and free oestradiol-17β appeared to decrease from about week 36 in both groups but these prepartum changes were not significant.

The proportion of cortisol circulating as free hormone was more variable than the other steroid hormones measured during pregnancy (4·47 ± 0·19%

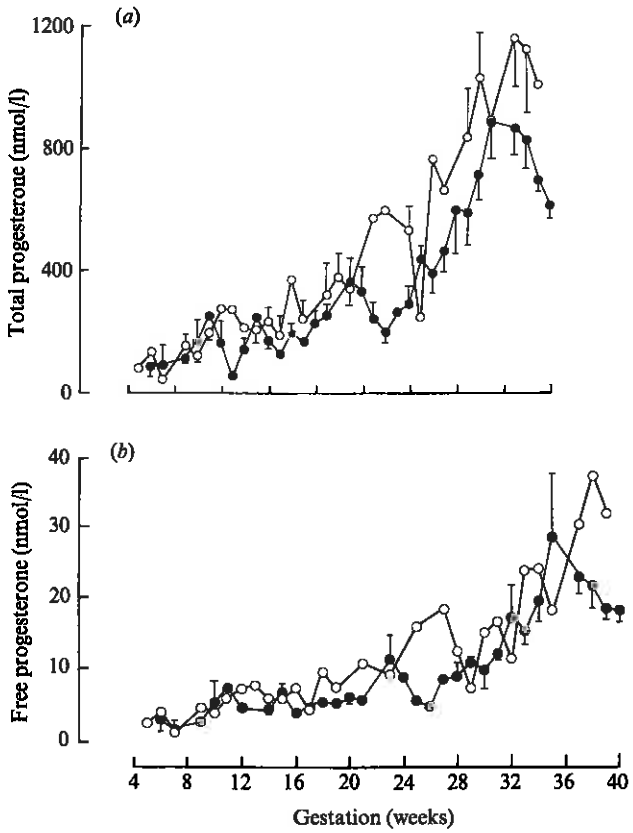

FIGURE 1. Plasma concentrations of progesterone in sub-fertile women who experienced an uneventful pregnancy (●) or who took medroxyprogesterone acetate up to week 18 of gestation for uterine bleeding (○). (*a*) Total (protein-bound plus free) progesterone, $n = 197$ and 69 individual determinations for untreated and treated women respectively. (*b*) Free progesterone, $n = 191$ and 64 individual determinations for untreated and treated women respectively. Values are means ± S.E.M. where $n > 3$.

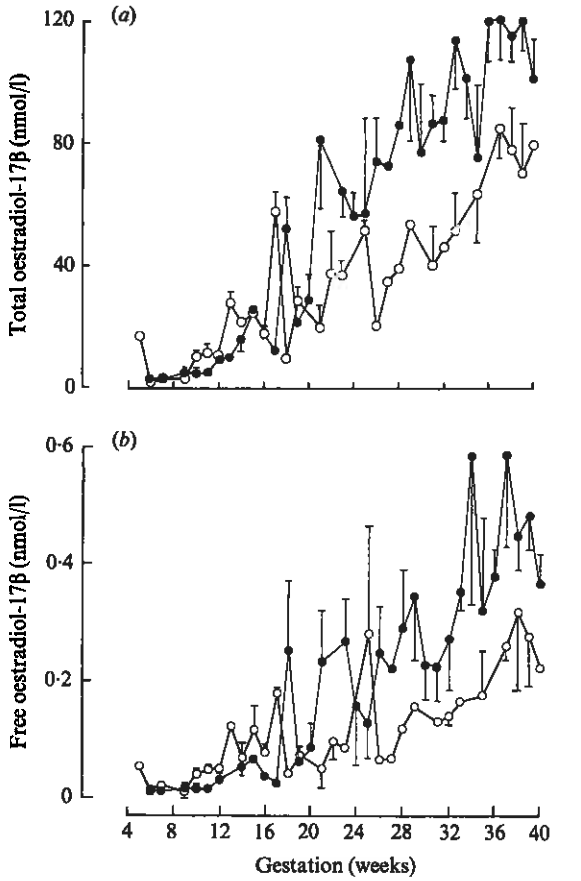

FIGURE 2. Plasma concentrations of oestradiol-17β in sub-fertile women who experienced an uneventful pregnancy (●) or who took medroxyprogesterone acetate up to week 18 of gestation for uterine bleeding (○). (*a*) Total (protein-bound plus free) oestradiol-17β, $n = 198$ and 70 individual determinations for untreated and treated women respectively. (*b*) Free oestradiol-17β, $n = 186$ and 66 individual determinations for untreated and treated women respectively. Values are means ± S.E.M. where $n > 3$.

(159) and 4·64 ± 0·20% (54) for groups A and B respectively). There was no significant difference in the free fraction of cortisol between the two groups of women so that the concentrations of total and free hormone in plasma increased similarly in both groups of women despite large individual differences (Fig. 3).

From weeks 16 to 18 the therapy was progressively withdrawn so that in plasma samples obtained after week 18 MPA was undetectable. The concentrations of total and free MPA in plasma samples obtained from weeks 5 to 16 from women in group B were 14·39 ± 2·30 nmol/l (54 samples) and 0·51 ± 0·13 nmol/l (43 samples) respectively. The free MPA fraction was 3·54 ± 0·22%.

DISCUSSION

This is the first report of total (protein-bound plus free) and free concentrations of progesterone, oestradiol-17β and cortisol measured in the same samples of maternal plasma collected from women throughout pregnancy. Previous studies of pregnant women who conceived spontaneously have shown that total concentrations of progesterone, oestradiol-17β and cortisol in maternal plasma rise progressively from 10 to 12 weeks of gestation to term (Tulchinsky *et al.* 1972; Allen & Lachelin, 1978; Mathur, Landgrebe & Williamson, 1980; Carr, Parker, Madden *et al.* 1981). In comparison, the 53 sub-fertile women in this study

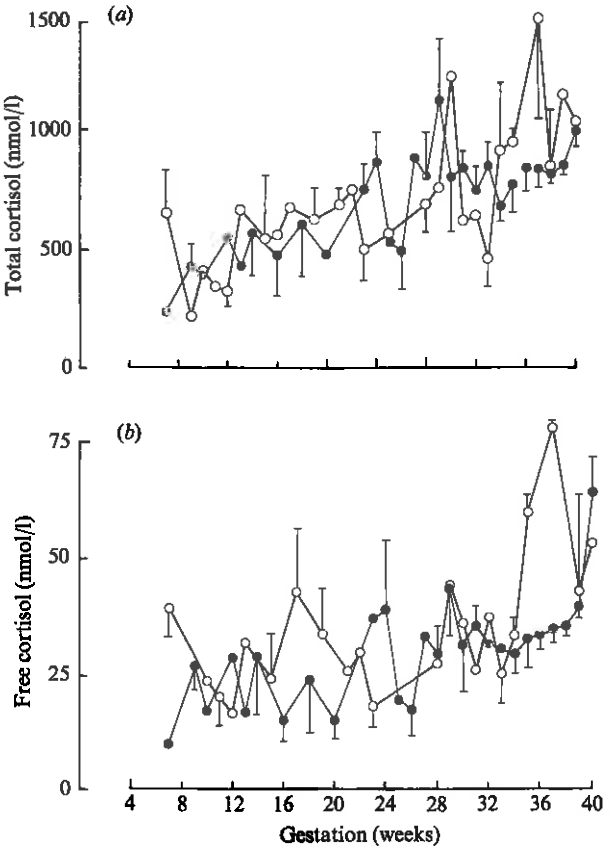

FIGURE 3. Plasma concentrations of cortisol in sub-fertile women who experienced an uneventful pregnancy (●) or who took medroxyprogesterone acetate up to week 18 of gestation for uterine bleeding (○). (*a*) Total (protein-bound plus free) cortisol, $n = 163$ and 58 individual determinations for untreated and treated women respectively. (*b*) Free cortisol, $n = 159$ and 54 individual determinations for untreated and treated women respectively. Values are means ± S.E.M. where $n > 3$.

who did not receive progestagen support during pregnancy (group A) did not display increased ovarian steroidogenesis from weeks 9 to 12 due to clomiphene or gonadotrophin therapy in their conception cycles. Mean total and free concentrations of progesterone in plasma appeared to decline after week 36 of gestation (Fig. 1), although this change was only statistically significant ($P < 0{\cdot}01$) for total hormone in group A. At the same time total and free concentrations of oestradiol-17β remained stable or even declined slightly (Fig. 2). These changes were not observed in all individuals.

It has been postulated that human labour is preceded by a withdrawal of the inhibitory effect of progesterone on the myometrium, possibly potentiated by an activating rise in oestradiol-17β (Csapo, 1961). Such pre-term changes have been observed only in carefully selected primigravida patients (Csapo, Knobil, van der Molen & Wiest, 1971; Turnbull, Patten, Flint *et al.* 1974). Studies on women from a wider obstetric base have failed to detect either a decline in progesterone or a rise in oestradiol-17β in the last few weeks of pregnancy (Eton & Short, 1960; Tulchinsky *et al.* 1972; Shaaban & Klopper, 1973; Turnbull, Anderson, Flint *et al.* 1977; Hartikainen-Sorri *et al.* 1981). Thus the significance of the decline in total and free progesterone concentrations observed here is unknown. It did not indicate the onset of premature labour since the women all delivered between weeks 37 and 41 of gestation.

Medroxyprogesterone acetate was given to 33 women who developed uterine bleeding up to week 6 of gestation. In 22 women (group B) bleeding stopped within 36 h of taking MPA and pregnancy proceeded uneventfully to term. The other 11 women aborted by the end of week 8. Progestagen support in pregnancy has been provided previously for women diagnosed as having inadequate luteal function, manifested either as a short luteal phase and/or slow rate of endometrial maturation. In these circumstances luteal progesterone secretion may be less than optimal for successful implantation of the embryo and maintenance of pregnancy before the beginning of placental progesterone synthesis (Dizerega & Ross, 1981). The efficacy of progestagen support therapy, whether in the form of progesterone or synthetic progestagens, in reducing the rate of spontaneous abortion remains controversial (Shearman & Garrett, 1963; Goldzieher, 1964; Klopper & MacNaughton, 1965; Fainstat & Bhat, 1983; Tognoni, Ferrario, Inzalaco & Crosignani, 1983). We do not wish to fuel this debate but note in this context that the women in group B received MPA for uterine bleeding very early in pregnancy. Luteal deficiency in terms of inadequate endogenous production of progesterone was not a criterion for treatment of these women with MPA.

The free fractions of progesterone, oestradiol-17β and cortisol did not vary significantly during pregnancy, either in the absence or presence of progestagen support. That is, their free hormone concentrations did not vary independently of their respective total hormone concentrations but reflected, at a lower level, changes in their total concentrations (Figs 1–3). This suggests that the maintenance of pregnancy by progesterone, feto-placental interaction in terms of oestradiol-17β synthesis, and maternal adrenal synthesis of cortisol did not involve a redistribution between inactive (protein-bound) and active (free) components of these hormones in maternal plasma. Furthermore, our finding of constant bound : free ratios of these hormones confirms the interpretations of large numbers of studies where changes in hormonal activity during pregnancy have been assessed by changes in total

hormone concentrations, determined by competitive protein-binding or immunoassay techniques. The amount of 5α-pregnanedione is reported to vary between 7 and 35% of the circulating concentration of progesterone during pregnancy and to either increase (Milewich, Gomez-Sanchez, Madden *et al.* 1975; Stoa & Bessesen, 1975) or decrease (Parker *et al.*, 1979) as pregnancy progresses. It is interesting that we obtained good correlation ($r = -0{\cdot}978$) between the direct and indirect methods of progesterone assay even though the progesterone antiserum cross-reacted with 5α-pregnanedione. This suggests that if 5α-pregnanedione interfered in our progesterone assay, leading to an overestimation of progesterone, then the ratio of the steroids remained constant throughout pregnancy.

We were interested in the total and free concentrations of progesterone and cortisol because both hormones are bound by transcortin, the concentration of which increases in maternal plasma as gestation progresses (Slaunwhite & Sandberg, 1959). In addition, the amount of circulating transcortin is increased in women receiving clomiphene therapy (Hammond, Radwanska & Talbert, 1980) and transcortin- and sex hormone-binding globulin are both synthesized in greater amounts as oestrogen output rises during pregnancy (Slaunwhite & Sandberg, 1959; Seal & Doe, 1967). Although the affinity of transcortin for progesterone increases relative to cortisol as pregnancy advances (Rosenthal *et al.* 1969), two factors appear to counterbalance this. First, the plasma content of albumin declines and, secondly, the concentration of cortisol increases relative to progesterone. The net result is that the proportions of free hormones did not change although their free concentrations increased (Figs 1*b* and 3*b*). Adrenal synthesis of cortisol was unaffected by uterine bleeding or exposure to MPA since total and free cortisol increased similarly throughout pregnancy in both groups of women (Fig. 3).

Total and free concentrations of progesterone were all significantly ($P<0{\cdot}005$) greater in group B than in group A. In contrast, until week 20 of gestation, no differences in total and free concentrations of oestradiol-17β between the groups were apparent. Subsequently, group B women had lower plasma concentrations of total oestradiol-17β ($P<0{\cdot}005$) and free oestradiol ($P<0{\cdot}01$) from 20 to 40 weeks of gestation. Threatened early abortion may be an event unrelated to the amounts of steroid hormone circulating in the latter half of pregnancy. However, these observations are paradoxical since group B women threatened to abort early in pregnancy presumably because of inadequate ovarian synthesis of steroids, especially progesterone. Greater placental synthesis of progesterone by the women in group B, especially in the second half of pregnancy, may reflect an intrinsic difference between the two groups.The placental weights were similar in both groups, indicating that the women in group B did not have a greater mass of steroidogenic tissue. Alternatively, exposure to MPA in the first trimester, or uterine bleeding itself, may cause placental progesterone synthesis to be increased or rates of metabolic clearance to be reduced. Medroxyprogesterone acetate depresses endogenous ovarian production of progesterone and oestradiol-17β (Johansson, 1971), possibly due to competitive inhibition of pregnenolone-3-ol-dehydrogenase-isomerase activity (Shinada, Yokota & Igarashi, 1978). The depression of ovarian progesterone synthesis in the luteal phase by MPA can be overcome by simultaneous administration of human chorionic gonadotrophin (Yovich, Stanger, Yovich & Tuvik, 1984). However, it is difficult to attribute either the increase in total and free progesterone or the decrease in oestradiol-17β concentrations in group B relative to group A to enzyme inhibition of either ovarian or placental steroidogenesis by MPA, since oestradiol-17β synthesis was not perturbed in the first half of pregnancy when ovarian synthesis predominates. Furthermore, MPA was undetectable in the plasma of group B women after week 20.

In conclusion, the biologically active free concentrations of steroid hormones do not vary independently of their total concentrations in maternal plasma during pregnancy. In contrast to women who do not threaten to abort early in pregnancy, women given progestagen support in the form of MPA to stop uterine bleeding in the first 6 weeks of gestation subsequently have higher circulating concentrations of total and free progesterone and lower concentrations of oestradiol-17β. At this stage it is not known whether the hormonal profiles of these women were different because of this therapy.

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