Interference with Biological Rhythm
A novel approach to metabolic disorders in women

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A Novel Approach to Metabolic Disorders in Women

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ABSTRACT

Women seem to be largely protected against certain 'welfare disorders', such as cardiovascular disease and osteoporosis, during their fertile years.

The metabolic changes observed during women's non-menstrual states, *i.e.* during pregnancy, after the menopause and during use of oral contraceptives, indicate the importance of sex steroids and an undisturbed biological rhythm. Treatment with monophasic, combined oral contraceptives constitutes a model for the non-cyclic state.

Growth hormone (GH) is a pituitary hormone that has major metabolic effects. The pattern of GH exposure to the target organ is of vital importance for the effects and changes in rhythm could possibly induce metabolic changes.

Growth hormone, cholecystokinin (CCK), osteocalcin and angiotensinogen were used as markers for metabolic effects and the concentrations in serum were recorded in women during non-menstrual states. The clinical material comprised a total of 60 women: 18 healthy non-pregnant, 25 pregnant, one lactating woman and 16 postmenopausal women. Using a portable pump and a non-thrombogenic venous catheter, blood samples could be collected at 30-min intervals during 24-h periods. Furthermore, the effects of estrogen and GH in the regulation of angiotensinogen were investigated in an experimental model in the rat.

Oral contraceptives were found to alter the secretion of GH towards a pattern of lower and more frequent peaks, though the total amount secreted during 24 h was unchanged. Oral contraceptives seem to induce a suppression of the 24-h concentrations of CCK, which may be important with respect to weight gain in some women. Osteocalcin in serum display a significant circadian variation. This emphasizes the need for careful timing of single point measurements and the value of continuous blood sampling. Oral contraceptives may reduce osteocalcin serum concentrations. The long-term effects on bone are unknown. During late pregnancy osteocalcin levels are extremely low, which could indicate osteoblast inhibition and reduced bone turnover.

The mode of GH administration is important for the plasma concentration of angiotensinogen in the non-pregnant rat. Estrogen effects on this protein may be mediated via a modification of GH secretion. Oral contraceptives not only increase angiotensinogen concentrations in serum but also markedly enhance their variability. Further studies are needed to elucidate the relation between the individual pattern of angiotensinogen and hypertension.
“It don't mean a thing if it ain't got that swing”
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Karlsson R, Edén S, von Schoultz B.  
*Altered growth hormone secretion during oral contraception.*  

II. Karlsson R, Lindén A, von Schoultz B.  
*Suppression of 24 hour cholecystokinin secretion during oral contraceptive use.*  
Am J Obst Gyn (In press).

III. Karlsson R, Edén S, von Schoultz B.  
*Oral contraception affects osteocalcin serum profiles in young women.*  

IV. Karlsson R, Edén S, Eriksson L, von Schoultz B.  
*Osteocalcin 24 hour profiles during normal pregnancy.*  
Gynecol Obstet Invest (In press).

V. Karlsson R, Rubin I, Stenlund H, von Schoultz B.  
*Increased variability in serum angiotensinogen levels during oral contraception.*  
Submitted for publication.

*Effects of growth hormone and estrogen on rat angiotensinogen quantified by an enzyme linked immunosorbent assay.*  
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ABBREVIATIONS

The following abbreviations are used in the text:

- ACTH: adrenocorticotropic hormone
- AI: angiotensin I
- bGH: bovine growth hormone
- BSA: bovine serum albumin
- CCK: cholecystokinin
- CRF: corticotropin-releasing factor
- GH: growth hormone
- ELISA: enzyme linked immunosorbent assay
- HA: human angiotensinogen
- HSA: human serum albumin
- hx: hypophysectomized
- PBS: phosphate buffered saline
- RAS: renin-angiotensin-system
- RIA: radio-immuno assay
- TRH: thyrotropin-releasing hormone
- TSH: thyroid-stimulating hormone
ABSTRACT

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Growth hormone, cholecystokinin (CCK), osteocalcin and angiotensinogen were used as markers for metabolic effects and the concentrations in serum were recorded in women during non-menstrual states. The clinical material comprised a total of 60 women: 18 healthy non-pregnant, 25 pregnant, one lactating woman and 16 postmenopausal women. Using a portable pump and a non-thrombogenic venous catheter, blood samples could be collected at 30-min intervals during 24-h periods. Furthermore, the effects of estrogen and GH in the regulation of angiotensinogen were investigated in an experimental model in the rat.

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Key words: Growth hormone, GH, cholecystokinin, CCK, osteocalcin, angiotensinogen, diurnal variation, oral contraception, pregnancy, menopause.
INTRODUCTION

Knowledge of the etiological mechanisms of disease is essential for the implementation of preventive measures and the initiation of health promotion strategies. In the past, interest has been focused mainly on diseases with short duration, mostly acute infectious diseases. The strategy for prevention was then to isolate the causative agent and to show that it caused the same disease in others, as postulated by Robert Koch in 1882. Once identified this often single causative agent could either be eradicated, or else people could be made less susceptible by vaccination. This strategy was then obviously successful and has since been an important part of our medical paradigm.

In more recent years, the focus has increasingly shifted to major, chronic diseases. The multifactorial etiology of modern 'welfare disorders' makes prevention more complex. The disappointing results in attempts to prevent ischaemic heart disease by treating high blood pressure (Collins et al., 1990) indicate that the elimination of one epidemiologically identified risk factor alone is not sufficient to constitute effective prevention. It implies the need to search for mechanisms linking risk factors with disease. Disorders such as hypertension, obesity and osteoporosis are evidently linked to genetic, environmental or lifestyle factors. Stress, a poor social network (Marmot & McDowell, 1986) and type A behaviour (Irvine et al., 1991) are such factors that have been discussed. Hypertension has also been suggested to be one of several manifestations of a metabolic syndrome, including non-insulin-dependent diabetes, dyslipidaemia and obesity (Reaven and Hoffman 1987; O'Hare 1988; Reaven 1988).

Women seem to be largely protected against certain 'welfare disorders' during their fertile years. The male/female ratio for diseases of the circulatory system is 3.5:1 in age group 45–54 years, falling to 1.3:1 after the age of 75 (WHO, World Health Statistics 1989). It has seemed natural to assume that the endogenous, ovarian steroids exert such a protective effect. There are three periods during the adult woman's life when this protection seems to disappear, viz. during pregnancy, after the menopause, and during treatment with oral contraceptives. Moreover is it a paradox that exogenous steroids as used for postmenopausal replacement may reduce osteoporosis and cardiovascular disease in postmenopausal women while similar steroids used as oral contraceptives in fertile women may cause hypertension and weight gain. These observations indicate that additional factors could be involved other than the sex steroid levels per se.

Sex steroids and metabolism

Cyclicity

In fertile women, concentrations of sex steroids in blood are characterized by cyclical changes. There are three periods during an adult woman's life when this cyclicity ceases viz. during pregnancy, after the menopause, and during use of oral monophasic, combined contraceptives. Well-known complications during preg-
nancy are hypertension, non-insulin-dependent diabetes and obesity (Tulchinsky & Ryan, 1980; Pritchard et al., 1985; Baird 1986). After the menopause there is an increase in the incidence of circulatory diseases as well as in osteoporosis with consequent fractures. Women using combined oral contraceptives run a greater risk than non-users of developing hypertension, thrombosis and myocardial infarction (Westerholm, 1980).

During oral contraception with estrogen-progestogen combinations, the normal cyclical changes in pituitary and sex hormone levels disappear to be replaced by a pattern of unchanging serum concentrations (Fig. 1).

The metabolic changes observed during women’s non-menstrual states, i.e. during pregnancy, after the menopause and during oral contraception, indicate the importance of an undisturbed biological rhythm. Treatment with monophasic, combined oral contraceptives is well suited as a model for a non-cyclical sex hormone state.

Effect mechanism

Sex steroids affect a wide variety of metabolic functions and this influence can be mediated principally in two ways: direct, via receptor binding in classical target organs such as the uterus and the vagina, or indirect by modifying the secretion of other hormones. Many of the metabolic changes caused by exogenous sex steroids are brought about by induced changes in liver metabolism. Estrogens increase the levels of several plasma proteins. The bile secretion is reduced and the blood coagulation is affected by increased levels of clotting factors. Although estrogen receptors are present in the liver, the concentration is only about one-half to one-

![Fig. 1. Estradiol and progesterone serum concentrations in women during the menstrual cycle, before and during oral contraception.](image-url)
third of that normally in the uterus (Eisenfeld & Aten, 1982). The liver differs from many typical target organs in that the steroids given are rapidly metabolized and processed. Thus, even though estrogen receptors have been identified and characterized in the liver, there must be serious doubts as to whether they have a physiological role in mediating estrogen action. This direct effect has recently been questioned and an indirect, modulating influence on other regulatory principles has been suggested (von Schoultz & Carlström, 1989). Animal experiments have clearly shown that sex steroid effects on hepatic drug and steroid metabolism are indirect and mediated via the hypothalamo-pituitary-liver axis (Mode & Norstedt, 1982).

The secretion of anterior pituitary hormones is pulsatile and the hypothalamus has been described as the pulse generating system. The factors regulating pituitary hormone release are peptides which are released from neurosecretory cells in the hypothalamus. As only very small amounts of these hypothalamic peptides are needed to cause the release of pituitary hormones, the system amplifies signals from the central nervous system, via the endocrine system, to affect virtually all cells in the body. Examples of such systems are the CRF-ACTH-corticoid and TRH-TSH-thyroid hormone systems. The hypothalamo-pituitary portal vascular system provides a unique connection between the central nervous system and the endocrine system (Fig. 2). This could be one effect mechanism linking environmental, lifestyle or behavioural factors with metabolic changes. It has been shown for many of the anterior pituitary hormones that not only the amount of hormone but also the precise pattern of secretion is of importance for the biological effects (Strand, 1983).

**Growth hormone secretory pattern**

Growth hormone (GH), is an anterior pituitary hormone which has anabolic
effects and stimulates protein synthesis in many tissues including bone, muscle, connective tissue and visceral organs (Merrimee, 1979). The biological action of GH is initiated by the binding of the hormone to specific receptors on the plasma membrane of the target cells. Several organs, including liver, heart, muscle and adipose tissue have high affinity binding sites for GH. In animal experiments the pattern of GH secretion has been clearly shown to be as important for the biological effect as the total amount secreted.

Most extensively studied is the sexual dimorphism, as the GH release is very different in male and female rats (Edén et al., 1987). In male rats GH, is released in large peaks separated by intervals with very low levels, whereas in females the pattern of GH release is less regular with smaller and more frequent peaks but with a higher basal level. These patterns can be mimicked in hypophysectomized experimental animals and a continuous GH-secretion has an estrogenlike ‘feminizing’ effect on a wide variety of metabolic functions (Eriksson et al., 1988a; Eriksson et al., 1989). The mechanism by which the target cells can distinguish between continuous ‘female’ and and intermittent ‘male’ hormone stimulation still needs clarification. The observation that the GH receptors are withdrawn from the plasma membrane, internalized to the Golgi membranes and then recycled to the plasma membrane, in time related to the GH pulses in male rats, has been suggested as one explanation to the pulse-related response (Bick et al., 1989).

The human GH plasma profile has a rhythmic circadian pattern where GH levels oscillate over a range of three orders of magnitude. GH secretion is also in Man sexually dimorphic. Women have generally higher and more diverse amplitudes, higher maxima and higher interpeak valleys than men (Fig.3). The age-related decline in parameters of total and pulsatile secretion is greater in women (Ho et al., 1987). The pulse frequency however is the same in women and men (Winer et al., 1990).

GH has profound effects on carbohydrate and lipid metabolism (Jansson et al., 1985) and acromegaly is often complicated by diabetes (Emmer et al., 1971). Many physical symptoms and clinical findings during normal pregnancy also bear a remarkable resemblance to certain well-known effects of growth hormone action.

![GH serum profiles from one young female (left) and one young male. (After Ho et al., 1987)](image-url)
Pregnancy has a clearly diabetogenic effect on the glucose metabolism (Kühl, 1975). GH and pregnancy both affect fat metabolism with an increase in plasma level of free fatty acids (Hollingsworth & Grundy, 1982). There are reports (Eriksson et al., 1988b; Frankenne et al., 1988) of the occurrence during pregnancy of a placental GH variant which, in contrast to pituitary GH, is secreted in a continuous fashion. During human pregnancy, starting at the end of the first trimester, a progressive change from episodic into continuous GH secretion seems to occur (Eriksson et al., 1988c), (Fig. 4).

In the present study the diurnal serum profiles of GH were studied in young women before and during oral contraceptive use. In an experimental model, the effects of hypophysectomy, various patterns of growth hormone administration and estrogens on serum levels of rat angiotensinogen were studied.

Weight gain

Obesity and hypertension are often parallel findings in patients with high cardiovascular risk and weight reduction is a generally accepted non-pharmacological treatment for hypertension (Tibblin and Åberg 1987; Berglund et al., 1989; Björkelund, 1990). Women tend to gain weight after pregnancy as well as after the menopause. Weight gain is also a well known side effect of hormonal contraception and especially of high dose progestogen-only methods. The mechanism is incompletely understood but a stimulation of appetite seems to be a major factor. Recent research has emphasized the role of neuropeptides in the regulation of appetite (Geracioli Jr et al., 1989). The neuropeptide cholecystokinin (CCK) has long been recognized as an agent in gastrointestinal physiology and its role in brain functions has been acknowledged as well (Vanderhaegen & Crawley, 1985). CCK is found not only in the gastro-intestinal tract but also, in comparable amounts, in the brain. In the study of food intake and body weight, the hunger mechanism has been extensively examined but it is still not known what stimuli

Fig. 4. Serum GH values during 24 h in one 25-year-old, non-pregnant woman, left and one woman during third trimester of pregnancy. (Pregnant woman, after Eriksson et al., 1988)
induce feeding (LeMagnen, 1985). Smith and co-workers (Gibbs and Smith, 1984) therefore suggested that one should study mechanisms of satiety rather than hunger. In the present study we monitored circadian variations of CCK before and during oral contraceptive use.

**Osteoporosis**

In postmenopausal women, osteoporosis with loss of bone density is an important factor in hip fractures.

The cause of postmenopausal bone loss is unclear but it is definitely linked to the change in sex hormone levels and the evidence that estrogens prevent bone loss and reduce the fracture rate is substantial (Stevenson & Whitehead, 1982).

Osteoporosis is a disorder characterized by low bone mass, with fragile bones and a tendency to fracture. The most common causes of bone loss are old age, immobility and the menopause. Less common but clinically important causes are corticosteroid medication, inactivity (Krolner & Toft, 1983), smoking (Baron, 1984), and pregnancy (Smith et al., 1985).

The main components of bone are cells, the organic matrix and minerals. Of central importance is the osteoblast, which is a specialized fibroblast, synthesizing bone collagen. It controls mineralization, receives and translates mechanical forces and modulates osteoclastic activity. The osteoclast, which resorbs bone, depends on the osteoblast for many of its actions. The skeleton is continuously removed by osteoclasts and replaced by osteoblasts. This bone turnover is most rapid in the young and when bone mass is constant, as in young adults, removal and replacement are precisely linked. Bone mass reaches a maximum at about age 30 and then declines as the cells ‘uncouple’ and osteoclasts begin to dominate. Women lose about a third of their cortical and half of their trabecular bone during a lifetime, whereas men lose two-thirds of these amounts. In osteoporosis of old age, osteoblasts fail to replace resorbed bone and make less bone matrix than normal (for ref., see Smith, 1987).

Osteocalcin is a bone matrix gamma-carboxyglutamate protein (bone Gla protein, BGP) synthesized by proliferating osteoblasts (Lian & Gundberg, 1988). A small fraction of newly synthesized osteocalcin is released to the circulation. Serum osteocalcin levels reflect bone turnover and have been suggested as a marker for bone mineral metabolism in postmenopausal osteoporosis and related skeletal disorders (Pødenphant et al., 1984; Stock et al., 1985; Ismail et al., 1988). Osteocalcin levels in serum increase after oophorectomy as well as following the menopause (Yasumura et al., 1987; Ismail et al., 1986). Previous studies have indicated that unopposed oestrogen as well as continuous estrogen-progestogen replacement may reduce osteocalcin levels (Christiansen et al., 1985; Riis et al., 1988).

The interpretation of data in this field and the evaluation of osteocalcin as a possible marker for sex hormone effects on bone metabolism is complicated by the fact that serum levels of osteocalcin may have a significant circadian variation (Gundberg et al., 1985). Osteocalcin levels in serum have been reported to vary almost two-fold over a 24-h period and previous reports, based on single blood samples obtained at lengthy intervals, must be interpreted with caution.
In the present investigation 24-h serum profiles of osteocalcin, with special emphasis on diurnal variations, were followed in healthy young women before and during treatment with oestrogen–progestogen combinations for oral contraception and during pregnancy. Osteocalcin serum levels were also recorded in postmenopausal women before and during estrogen replacement therapy.

**Hypertension**

Cardiovascular disease is a major health problem in the industrialized world and myocardial infarction has become a leading cause of death during this century (WHO MONICA Project, 1987; Epstein, 1988). Hypertension is one of the major risk factors for coronary heart disease and in women a known complication to both pregnancy and combined oral contraception.

The renin-angiotensin-system (RAS) plays an important role in the regulation of blood pressure. The system is basically an enzyme-substrate reaction where the enzyme is renin, synthesized in the juxtaglomerular apparatus, and the substrate is angiotensinogen, synthesized in the liver. The first product, angiotensin I, is then further converted via converting enzyme to the active pressor substance, angiotensin II. (Fig. 5).

Angiotensinogen (renin substrate) is a glycoprotein with a molecular weight of about 60,000, synthesized mainly by the liver and the only known precursor of the angiotensin peptides (Poulsen, 1973; Ménard et al., 1983). These peptides play an important role in the control of blood pressure via the RAS (Reid et al., 1978). It is well established that serum concentrations of angiotensinogen are rate limiting with respect to the renin-angiotensinogen reaction during normal physiological conditions (Gordon, 1983; Jaramillo et al., 1987). Glucocorticoids and insulin are known stimulants of angiotensinogen synthesis and a four-fold increase in serum levels also occurs during normal pregnancy (Hasegawa et al., 1973; Dzau & Herrmann, 1982). Furthermore, estrogen administration has been reported to

![Fig. 5. Components of the renin-angiotensin system (After Roberts., 1981).](image-url)
enhance liver angiotensinogen synthesis (Eisenfeld et al., 1973; Krakoff & Eisenfeld, 1977). However, the quantitative increase in angiotensinogen which occurs under these conditions is similar in women who become hypertensive and in those who remain normotensive (Roberts, 1981; Shionoiri et al., 1983). Little is known about temporal variations in circulating angiotensinogen in normotensive or hypertensive women. In the present study 24–h serum profiles of angiotensinogen were monitored before and during oral contraceptive use. Furthermore, the effects of estrogen and growth hormone in the general, metabolic regulation of angiotensinogen were investigated in an experimental model in the rat.
AIMS OF THE STUDY

The aims of the present study were:

• to study the secretory pattern of GH and CCK, as reflected by serum profiles, in individual women before and during oral contraception;

• to investigate the effects of pregnancy, menopause and oral contraception on the serum level and diurnal variation of osteocalcin;

• to study the effect of oral contraceptives on serum levels and variability of angiotensinogen;

• to elucidate the impact of continuous and intermittent GH administration and estrogen upon circulating angiotensinogen levels in an experimental model;
MATERIAL AND METHODS

Subjects

The clinical material comprised a total of 60 women. Ten young women, attending the local health care centre for contraceptive counselling, were recruited for continuous, diurnal blood sampling. Their mean age was 25 (range 22–31) years. All were apparently healthy with regular menstrual cycles and they received no other drugs before or during the experiment. One of these women was later excluded due to technical problems during sample collection. In the remaining 9 women, 24–h serum profiles of GH, CCK, osteocalcin and angiotensinogen were established. The women were investigated before and after 2–3 months of treatment with monophasic, combined oral contraceptives (30 μg ethinyl-estradiol/150 μg desogestrel or 30 μg ethinylenestradiol/150 μg levonorgestrel).

Five women studied in gestational weeks 17, 34, 37, 38 and 38 had a normal pregnancy with vaginal term delivery of a healthy infant of normal birth weight. In two women, studied in weeks 11 and 15, pregnancy was terminated by legal abortion. In addition, the diurnal profile of osteocalcin was established in one lactating woman 4 weeks after delivery.

Sixteen postmenopausal women, mean age 56 (range 50–65) years, with typical climacteric complaints were also included. They were apparently healthy and had taken no drugs for at least 3 months. All had elevated FSH serum concentrations (>25 U/l) and were more than 12 months postmenopausal. In 15 women venous, blood samples were drawn before and after 1 and 6 months of continuous replacement therapy with 2 mg estradiol/1 mg norethisterone daily. In one woman a complete 24–h plasma profile for osteocalcin before and after 2 months of treatment with unopposed estradiol valerate 2 mg daily was recorded.

Eight healthy, non-hypertensive women and 18 pregnant women during the first trimester were recruited for single venous blood samples for determination of angiotensinogen.

The study was approved by the local medical ethics committee and all women gave their informed consent to participate.

Sample collection

Venous blood samples for the 24 hour profiles were obtained using the non-thrombogenic blood withdrawal system ad modum Kowarski et al., (1971). Continuous sampling from an antecubital vein via heparin-coated PVC tubing (Durascan Medical Products ApS, Odense, Denmark) was effected by means of a portable, battery charged eccentric pump (Cormed, Medina, N.Y., USA). During 24 h, collecting tubes were changed at 30-min intervals and approximately 5 ml was collected in each 30-min period. The sampling technique with a portable unit was adopted to monitor diurnal variations during ‘normal’ daily activities and reasonably physiological conditions (Fig. 6). In some of the women recruited technical problems occurred during the 24 h of sampling. Most frequent was
repeated blood clotting, perforation of vessels or various forms of discomfort. This, in some cases, caused incomplete profiles but only one woman had to be excluded due to technical problems. The activity and food intake of subjects was recorded but in no way restricted. Walking and daily activities including housework and moderate exercise were encouraged and the subjects were allowed to retire after 10 p.m. The period of sleep was recorded by observation by the investigators. Serum was separated after centrifugation and stored at −20°C until assay.

Assays

All samples were assayed ‘blind’ in duplicate. Individual samples from each subject were analysed in a single assay.

Growth hormone

Serum concentrations of hGH were determined by an immuno-radiometric technique using a commercial kit obtained from Pharmacia AB, Uppsala Sweden. The intra-assay and inter-assay coefficients of variation were 5.4% and 8.8% respectively. The minimum detectable concentration was 0.3 mU/l.

Cholecystokinin

Serum was partially purified by adsorption on Sep-Pak-C18 cartridges (Waters Associates, Milford, Mass, USA) and elution of the fraction containing CCK with acetonitrile: 0.1% acetic acid 1:1. The eluates were evaporated to dryness and reconstituted in assay buffer and analyzed by radio-immunoassay (Himeno et al., 1983). Within and between assay coefficients of variation were 4.0% and 10.6%.

Fig. 6. Continuous venous blood sampling carried out by means of a non-thrombogenic catheter, heparin-coated PVC tubes and a portable pump. The pump was carried in a small bag and the participating women could take part in fairly normal daily activities.
The detection limit of the standard curve was 2 pM; however, concentration of samples by Sep-Pak solid extraction enabled detection at 0.2 pM.

Osteocalcin
Concentrations of osteocalcin in serum were determined by radio-immunoassay using a commercial kit (OSTK-PR) obtained from Compagnie Oris, Gif-sur-Yvette, France. The sensitivity of this assay was 0.35 ng/ml and intraassay and interassay coefficients of variation 8% and 9%.

For control purposes, serum concentrations of osteocalcin in a fraction of the serum samples were also determined by an alternative radio-immunoassay using a commercial kit (Osteocalcin RIA) obtained from Incstar Corp. Stillwater, Minn. The sensitivity of this assay was 0.2 ng/ml and intra-assay and interassay coefficients of variation 7% and 10%.

While the concentrations read by the Osteocalcin RIA were on average 50% lower than values obtained by the OSTK-PR assay, the correlation between the two methods was excellent, r=0.97 (Fig. 7).

Angiotensinogen
Direct determination of human angiotensinogen (HA) concentrations was performed by an enzyme linked immuno-sorbent assay (ELISA). Immunoplates (Nunc, Roskilde, Denmark) were coated with a monoclonal HA antibody, F1A1, (Rubin et al., 1988) 3 μg/ml in 10 mmol/l phosphate buffer (PBS), pH 7.4, 0.15 mol/l NaCl, using 125 μl/well. The plates were incubated overnight at +4°C. The wells were blocked with 1% bovine serum albumin, (BSA, Behring Werke, Germany) in PBS, 200 μl/well, for 1 h at room temperature. Semipurified HA was used for the standard curve. The concentration of the standard HA was determined by radio-immunoassay after total degradation by excess renin as described below.

Human sera were added at appropriate dilutions in PBS/0.1% BSA. 100 μl of calibrator and/or sample was added and incubated for 1 hour. After washing, the plates were incubated with polyclonal rabbit anti-HA, diluted in PBS/0.5% BSA

![Graph](https://example.com/graph.png)

**Fig. 7.** Correlation between concentrations of osteocalcin in 48 serum samples measured by two different radio-immunoassays.
for 1 h. Following washing, wells were incubated for 1 h with 100 μl peroxidase labelled goat anti-rabbit immunoglobulin (Tago, USA) diluted in PBS/0.5% BSA. After addition of 100 μl substrate buffer, enzyme reaction was allowed to proceed for 2–19 min. The reaction was stopped by adding 100 μl 2.5 M sulphuric acid. Optical density was read at 492 nm on an ELISA reader, EAR 400T (SLT-Lab instruments, Austria). Washing between each step was performed using PBS/0.5% Tween 20. All incubations were performed at room temperature on a rocking platform, 4 cycles/sec. The intra-assay and interassay coefficients of variation were 2% and 8% respectively.

Indirect determination was made by radio-immunoassay (RIA) of angiotensin I (AI) generated after incubation with excess human renin. 5 μl human serum was mixed with 25 μl human renin, diluted in TRIS/HCL buffer, 0.2 mol/l, pH 7.5, with 0.3% human serum albumin, HSA (Behring Werke, Germany), RIA-buffer. The human renin was prepared as described by Haas et al., (1966). Each vial contained rabbit anti-AI and 1.2x10^-3 Goldblatt units of human renin. The reaction was allowed to proceed for 2, 3 and 4 hours at 37°C and stopped by cooling to 0°C (icebath). 125I-AI was added in 1 ml RIA-buffer and equilibrium was obtained after 18 h incubation at +4–5°C. Free AI was removed with charcoal coated with Dextran T70 (Pharmacia, Sweden) and centrifugation. The antigen-antibody complex was counted in a gamma-counter (Hydrogamma, USA). The intra- and inter-assay variation was 7 and 9% respectively.

Each sample was investigated twice in ELISA and RIA on two different days. The samples were tested in duplicate in at least two different dilutions. The results obtained by ELISA were closely correlated to those obtained by RIA (r=0.97).

**Serum total protein**

Total protein determinations were made according to Grove et al., 1968, using purified human serum albumin, HSA(Behring Werke, Germany), as a standard. The detection limit was 1 μg protein/ml.

**Rat angiotensinogen**

The purification of rat angiotensinogen (Bouhnik et al., 1982) made it possible to develop an ELISA for rat angiotensinogen. This ELISA was based on mono- and polyclonal antibodies against rat angiotensinogen (Clauser et al., 1984). 96-well microtitre plates (Nunc, Roskilde, Denmark) were coated with 200 μl of 10 μg/ml monoclonal antibody to rat angiotensinogen (Mab 136) in 0.1 M NaHCO₃. The plate was incubated overnight at room temperature and washed three times with 1% NaCl, 0.05% Tween-20. Serially diluted (1:3) pooled rat serum was used to determine the relative concentrations of rat angiotensinogen. The reference serum and the samples were both diluted in PBS containing 0.05% Tween-20, 0.05% BSA and 0.01% merthiolate. All determinations were made in duplicate. Samples to be assayed were diluted 1:5000 in the same buffer and were incubated on the plate for 2 h. After washing, a rabbit anti-rat angiotensinogen antiserum was added in a dilution of 1:20,000 for 2 h. Finally, an enzyme-linked goat antirabbit IgG (Bio-Rad, Richmond, Calif.), diluted 1:5000, was added for 2 h. After washing, 2 mg 1.2-phenylenediamine (Dakopatts, Copenhagen, Denmark) was dissolved in 50 ml 0.1 M citric acid-phosphate buffer, pH 5.0, 5 μl 30% H₂O₂ was added and
200 μl of this solution was added to each well. Absorbance was measured in a Titertek Multiscan at 450 nm.

The ELISA was technically simple to handle and a standard curve is shown in Fig. 8. Standard dilutions of pooled rat sera ranging 1:100 to 1:218,700 were used and the working dilution for samples was 1:5,000. The affinity constant of the first monoclonal antibody (Mab 136) was previously determined to 3.8×10^{-10} M. The intra- and interassay variations were 5% and 16% respectively.

Animal experiments

Animals

Sprague-Dawley rats were purchased from Alab Laboratories Ltd, Stockholm, Sweden and maintained under controlled conditions of constant temperature (24–26°C), humidity (50–60%) and a 14 h light: 10 h dark cycle (lights on at 6 a.m.). Standard laboratory chow (Type R 3: Ewos, Södertälje, Sweden) and tap water were freely available. Female rats were hypophysectomized by the standard parapharyngeal approach at 50 days of age. Completeness of hypophysectomy was determined by recording weight gain during a 1-week observation period before starting replacement therapy. Animals gaining more than 0.5 g/day in body weight were excluded. Ether was used as an anaesthetic when osmotic minipumps were implanted and when intramuscular injections were given. For comparison, intact, untreated male rats were used as a control group. The animals were killed by decapitation after 7 days of treatment and trunk blood was collected in chilled heparinized tubes. Plasma was separated and stored at −20°C until assayed.

Fig. 8. An enzyme linked immunosorbent assay for rat angiotensinogen. Standard dilutions (1:9) of pooled rat sera ranging 1:100 to 1:218700 were used and the working dilution for samples was 1:5000.
Hormonal treatment

Recombinant bovine growth hormone (bGH; American Cyanamide Co., Princeton, N.J. U.S.A.) was diluted in 0.05 M phosphate buffer (pH 8.8) with 1.6% glycerol and 0.02% sodium azide and given in a daily dose of 1 mg/kg/day. Administration of bGH was performed either as two daily injections at 12 h intervals in volumes of 0.2 ml s.c. or as a continuous infusion by means of Alzet osmotic minipumps (Alza Corp, Palo Alto, Calif.) implanted s.c. on the back of the animals. The minipumps had a filling volume of 240 μl and an estimated pumping rate of 1 μl/h at 37°C (Fig. 9).

Estradiol valerate (Progynon Depot: Schering, Berlin, Germany) was diluted in sesame oil and 0.2 ml (1.5 mg/kg/15 days) was given i.m. as a single injection. All hypophysectomized rats received replacement therapy with L-thyroxine (Sigma Chemical Co., St Louis, Mo, U.S.A.; 10 μg/kg/day) and hydrocortisone acetate (Hydrocortial Organon Ltd, Göteborg, Sweden; 500 μg/kg/day). L-thyroxine and hydrocortisone acetate were diluted with saline and given in a volume of 0.2 ml s.c. at 8 a.m. daily. The rats were treated according to six different protocols as shown in Table I.

Analysis of the plasma profiles and statistical methods

The pattern of peaks in the GH profiles was analysed by the PULSAR program (Merriam & Wachter, 1982). The assay coefficients of variation were calculated

Fig. 9. Continuous bGH infusion was accomplished by means of an osmotic minipump implanted s.c. on the back of the animal.
from the results of 10 repeated assays of three plasma pools. The parameters \( G(1) \) to \( G(5) \) of the PULSAR program were set to \( G(1) = 6.5 \), \( G(2) = 5.2 \), \( G(3) = 3.8 \), \( G(4) = 3 \), and \( G(5) = 2.4 \) in accordance with Hindmarsch et al., (1987). The smoothing time was set to half of the total profile time. The splitting cut-off parameter was set to 2.7. The weight assigned to peaks was 0.05.

For each profile the following parameters were extracted: overall mean GH concentration, mean of the baseline concentration, mean peak amplitude, mean peak duration, peak area, and interpeak interval.

The serum profiles for human angiotensinogen were analysed using another computer program, MESOSAUR (Systat Inc., Evanstone, Ill., USA). Here, a single peak was defined as a deviation from the mean of the serial subject values exceeding the 95% confidence limit. A peak in time-series, as calculated from the successive relative changes \( \frac{y_t}{y_{t-1}} \), was equal to a shift in level.

The differences between groups of rats were tested by the Kruskal-Wallis test. The effect of treatment with oral contraceptives on CCK values was evaluated with two factor ANOVA with repeated measures, the effect on angiotensinogen was evaluated with the sign test and the effect on GH with the non-parametric Kruskal-Wallis test. The differences in osteocalcin serum levels in the post-menopausal women after replacement therapy were assessed by Wilcoxon signed rank test.

Table I Treatment regimen and number of animals in the different experimental groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sham</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>Hx only</td>
<td>7</td>
</tr>
<tr>
<td>C</td>
<td>Hx+E2</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>Hx+bGH 2 s.c. injections/day</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>Hx+bGH continuous infusion</td>
<td>7</td>
</tr>
<tr>
<td>F</td>
<td>Intact males</td>
<td>9</td>
</tr>
</tbody>
</table>

Hx= hypophysectomy; E2 = estradiol-17β; bGH= bovine growth hormone
RESULTS AND COMMENTS

Altered growth hormone secretion during oral contraception (I)

In the present study, ethinyl estradiol was given together with a progestogen for oral contraception in 9 healthy young women. Before treatment, all women showed typical GH plasma profiles with intermittent peaks (mean value 6 peaks /24 h) with a duration of 1–3 h. Between peaks basal levels were low in most women and often below the detection limit of the assay. The overall mean GH concentration displayed considerable individual variations ranging 1.0–8.2 mU/l. During treatment with oral contraceptives, significant effects on the 24–h plasma profile of GH were observed. There was a shift towards smaller peaks at a higher frequency. Fig. 10 illustrates this effect in one individual woman.

For the whole group the mean peak amplitude decreased from 11.2 to 7.3 mU/l (p<0.05) and the mean peak area from 24.1 to 14.1 mU/l (p<0.05). The higher frequency was reflected by a reduced interpeak interval from 4.3 to 3.0 h (p<0.05). There were no significant effects on the overall mean GH concentrations, baseline concentrations, or peak duration.

The significance of this change in young women taking oral contraceptives is unclear, but animal experiments have emphasized the importance of pituitary function and of growth hormone secretion in the regulation of various aspects of

![Graph showing serum GH levels in a 28 year old woman before and during oral contraception with 30 µg ethinylestradiol/150 µg desogestrel.](image)

Fig. 10. Serum GH levels in a 28 year old woman before and during oral contraception with 30 µg ethinylestradiol/150 µg desogestrel.
liver metabolism (Colby, 1980; Jansson et al., 1985). Estrogen treatment is well known to stimulate GH secretion in both men and women (Wiedeman et al., 1976; Hindmarsch et al., 1987; Stege et al., 1987). The GH secretory response to various stimuli is also enhanced by estrogen administration (Dawson-Hughes, 1986). The administration of even a very low dose of ethinyl estradiol (100 ng/kg, day) resulted in a significant augmentation of pulsatile GH activity in prepubertal girls with Turner’s syndrome (Mauras & Rogol, 1989).

Hypertension, venous thromboembolism and other cardiovascular disorders are potential side effects of oral contraceptives which may be related to enhanced hepatic action following exogenous sex steroid administration (Gilmer, 1989). Alterations in protein synthesis, coagulation factors and lipid metabolism are of obvious interest but the precise mechanism regulating these changes is poorly understood (von Schoultz & Carlström, 1989). Sex hormone action on liver metabolism is intimately related to pituitary function (Colby, 1980).

Certain liver effects of oral contraceptives, viz. on the synthesis of renin substrate, high density and low density lipoproteins, various coagulation factors and anti-thrombin III, might possibly be related to a sex steroid induced change in GH secretion during treatment rather than consequences of a direct steroid action on hepatic cells. Indeed there are data to support such a concept for the estrogen component of oral contraceptives (Mode & Norstedt, 1982; Jansson et al., 1985; Jellinck et al., 1985; Edén et al., 1987; Nilsson et al., 1988). Currently, there is virtually no information how different progestogens affect GH secretion and the present material was too small to allow a comparison between levonorgestrel and desogestrel in this respect.

Although oral contraceptives have been widely used for many years there is still considerable uncertainty about the regulatory mechanisms involved and the significance of many of the metabolic changes that have been observed when these drugs are administered. The finding that oral contraceptives may alter GH secretion in healthy young women opens one new avenue for further research.

**Suppression of the gut-brain peptide and satiety factor cholecystokinin during oral contraceptive use (II)**

Before treatment mean values for serum levels of CCK ranged 1.4-2.4 pM during the 24-h period of observation. During treatment mean values ranged 0.7 to 1.8 pM. The profile values for one individual woman are given in Fig. 11.

At all points throughout the 24 h period, mean CCK values were lower when women were using oral contraceptives (overall effect p<0.001).

Animal experiments have clearly demonstrated the satiety-inducing effects of peripherally and centrally administered CCK. The use of recently developed specific CCK receptor antagonists has provided evidence that endogenous CCK plays a physiological role in food intake regulation. CCK suppresses food intake not only in experimental animals but also in humans. Recent studies have shown disturbances in meal-related CCK secretion in eating and affective disorders (Geracioti Jr et al., 1989). Cyclical mood-changes, as in the premenstrual tension syndrome, have been reported during estrogen-progestogen therapy. Disturbances
in mood regulation are frequently associated with alterations in appetite and food intake.
The present results clearly demonstrate a suppression of unstimulated 24-h serum levels of CCK during oral contraception, which might well relate to increased appetite and weight gain in individual women.

Diurnal fluctuations of osteocalcin and lower levels during sex hormone therapy (III)

The complete 24-h profiles of serum osteocalcin in one 31-year-old woman before and after 2 months of oral contraception with 30 μg ethinyl estradiol/150 μg desogestrel are illustrated in Fig. 12.

The overall pattern was the same in all women using oral contraceptives. There were striking point to point fluctuations, in the range 0.5–10.0 ng/ml and peak levels were generally reached nocturnally. The suppressive influence of hormone therapy upon serum osteocalcin levels was obvious. The irregular activity was maintained but at a lower level. Almost all individual values obtained at 30 min intervals for 24 h were lower after treatment. Before treatment the overall 24-h mean value was 2.9 ng/ml (range 1.2–4.3 ng/ml). After 2–3 months of oral contraceptive use the corresponding figures were 1.5 ng/ml and 0.8–2.8 ng/ml. For the whole group the mean osteocalcin concentration decreased by 1.4 ng/ml (p<0.01) during treatment.

The 24-h serum profiles for osteocalcin in a 65-year-old woman are shown in Fig. 13. The irregular diurnal peak activity in this postmenopausal woman was quite similar to that recorded in younger women. When 2 mg of unopposed estradiol valerate was given as replacement therapy for 2 months, the same

![Figure 13](image_url)

Fig. 13. Serum osteocalcin serum levels in one 65 year old woman before (—o—) and during (---o---) replacement therapy with 2 mg unopposed estradiol valerate.
suppression of circulating osteocalcin levels as with combined estrogen–progestogen treatment occurred. The mean value±SD before treatment was 1.55±0.53 ng/ml, as compared with 0.96 ±0.37 ng/ml during therapy.

Mean values (±SEM) for osteocalcin in single blood samples from 15 postmenopausal women during estrogen–progestogen replacement therapy are illustrated in Fig.14. Already after the first month of treatment a 15% reduction was observed in the group mean value (p<0.05); after 6 months the reduction was 30% (p<0.01).

By these serial single-point measurements, we confirmed the previous finding (Civitelli et al., 1988; Riis et al., 1988) that combined estrogen–progestogen replacement therapy will suppress the mean osteocalcin level in a group of postmenopausal women.

In the present study, complete 24-h profiles for osteocalcin concentrations in serum before and during estrogen–progestogen therapy were established for individual women for the first time. The continuous blood sampling technique made it possible to follow osteocalcin levels in non-hospitalized women during fairly normal daily activities. Marked fluctuations in osteocalcin concentration were found during the 24-h sampling period. The values also displayed an apparent circadian rhythm. Day-time values were on average lower than nocturnal concentrations. Although the number of subjects was quite small and only single 24-hour periods were investigated, these data agree closely with a previous report where osteocalcin levels were found to be low in the morning, reach a nadir around noon and then rise markedly to reach a maximum around 4 a.m. (Gundberg et al., 1985). Recent data, based on single blood samples, have suggested that osteocalcin might be used as a marker for bone turnover during estrogen therapy in postmenopausal women (Pødenphant et al., 1984; Stock et al., 1985; Civitelli et al., 1988; Riis et al., 1988; Garcia-Carrasco et al., 1988; Johansen et al., 1988). Therefore, the finding that osteocalcin levels were markedly depressed

![Fig. 14. Mean values (±SEM) for osteocalcin in single blood samples from 15 postmenopausal women before and after 1 and 6 months of replacement therapy with 2 mg estradiol/1 mg norethisterone acetate daily.](image-url)
also in young women receiving estrogen–progestogen combinations for contraception, is important.

The suppressive effect of postmenopausal estrogen–progestogen replacement therapy on osteocalcin levels is well known. The high serum levels of osteocalcin observed in postmenopausal osteoporotic women are believed to reflect increased bone turnover secondary to enhanced bone resorption. High osteocalcin levels have been associated with a net loss of bone (Ismail et al., 1986). It is generally believed that estrogens may reduce bone resorption, increase bone mass and secondarily reduce bone turnover and also osteocalcin levels (Christiansen et al., 1985; Civitelli et al., 1988; Johansen et al., 1988). Whether the same effect mechanism is relevant also for the present finding that even in young women receiving estrogen–progestogen combinations for contraception, osteocalcin levels were markedly depressed, is uncertain. Hormones, such as vitamin D and thyroxin, are important for osteoblast activity and hence osteocalcin synthesis. Treatment with oral contraceptives might reduce the free biological active fraction of these hormones as a consequence of increased protein binding in plasma (Laurell & Rannevik, 1979).

**Extremely low levels of osteocalcin without diurnal fluctuations during late pregnancy (IV)**

The impact of gestation on maternal bone and mineral metabolism is poorly understood. During human pregnancy, highly increased concentrations of calcitonin and PTH are recorded and a massive transplacental transfer of calcium and phosphorus to the growing foetus occurs (Pitkin, 1985; Cole et al., 1987). Whether these physiological changes significantly affect maternal bone mass is unclear. Studies by photon absorptiometry have yielded conflicting results (Goldsmith & Johnston, 1975; Christiansen et al., 1976; Lambke & Brundin, 1977). Evidence has been presented that osteomalacia may occur in some pregnant women with a low calcium intake (Rab & Basier, 1976) and in one animal study a slight decrease in bone matrix size was reported (Mbuyi-Muamba & Dequeker, 1984). However, the net effects of gestation on maternal bone density and mineral metabolism are largely unknown. The 24-h profiles for osteocalcin in serum from 6 of the 12 women investigated are presented in Fig. 15.

The overall pattern in the 4 non-pregnant women was quite similar. There were striking fluctuations during the 24–h sampling period, values ranging 0.5–4.0 ng/ml. The one woman investigated in the 11th week of pregnancy displayed a similar 24-h osteocalcin plasma profile, whereas in the woman in the 15th, and notably in the woman in the 17th, week values were much more stable and lower, ranging 0.8–1.8 mg/ml and 0.2–0.5 ng/ml respectively. The results obtained from women during late pregnancy were in marked contrast to those from non-pregnant women. Osteocalcin serum levels were generally very low (range 0.2–0.4 ng/ml) and often below the detection limit of the assay and there was no apparent diurnal variation. In the lactating woman osteocalcin serum levels were also low (0.3–0.8 ng/ml) and stable.

Previous studies on osteocalcin levels in pregnancy have produced conflicting
results. Cole et al., (1987) found that serum levels declined during mid-pregnancy, returning to normal non-pregnant levels in the third trimester. Rodin et al., (1989) found a complete disappearance of osteocalcin from the circulation after the first trimester, values were below the detection limit of the assay but returned to normal shortly after delivery. The interpretation of data on osteocalcin is complicated by the fact that serum levels display a significant circadian variation (Gundberg et al., 1985). In both the previous studies in pregnant women, only single samples were analysed. In the present study, 24-h serum profiles of osteocalcin were established for individual women during different stages of normal pregnancy.

Although the clinical material was small the disparity between late gestation and the non-pregnant state was apparent in all women investigated. Osteocalcin concentrations in late gestation were clearly reduced and even below the limit of

Fig. 15. Serum concentrations of osteocalcin during 24 hours in six individual women. One non-pregnant, one lactating and four during different stages of pregnancy. The shadowed area indicates levels below the detection limit of the assay.
detection and there was no evidence of a diurnal variation. These data could possibly reflect a decrease in bone turnover and a reduced osteoblast activity, which might well be a physiological adjustment to pregnancy.

Previous studies have shown that unopposed estrogen, as well as combined estrogen/progestogen replacement may reduce osteocalcin levels (Stock et al., 1985; Riis et al., 1988).

In postmenopausal osteoporosis increased concentrations of osteocalcin are believed to reflect increased bone turnover secondary to enhanced bone resorption. During pregnancy, a high level of osteoblastic activity would obviously counteract the massive transplacental transfer of calcium and phosphorus needed by the growing foetus (Pitkin, 1985; Cole et al., 1987). Whether significant bone resorption actually occurs during pregnancy is not known, but osteoblast inhibition and consequently low osteocalcin levels would nevertheless seem favourable during pregnancy.

The precise mechanisms which regulate osteocalcin concentrations in serum are unknown. Estrogen treatment is known to affect GH secretion and possibly high concentrations of circulating estrogens could be involved in the regulation of osteoblast activity (Dawson-Hughes, 1986; Duursma et al., 1986). Osteocalcin serum levels have been found to increase during treatment with human GH in GH-deficient adults and to decline in acromegalic patients following surgery (Sidenius-Johansen et al., 1990). During pregnancy there is a gradual decline in pituitary GH serum concentrations and, during late gestation, levels are very low or even undetectable. A placental GH variant which, in contrast to pituitary GH, is secreted in a continuous fashion has been reported (Eriksson et al., 1988; Frankenne et al., 1988). It is interesting to note in this context that the osteocalcin serum profile in the one woman investigated during early pregnancy displayed similar characteristics as in non-pregnant women, whereas during late pregnancy there was a marked change towards a very stable pattern of generally low or undetectable osteocalcin concentrations.

To conclude, continuous 24-h measurements seem to confirm previous observations (Rodin et al., 1989) based on single blood samples, that during late pregnancy, maternal levels of osteocalcin in serum are extremely low or even absent. Osteocalcin should therefore be of little value as a marker for bone and mineral metabolism during pregnancy.

Low osteocalcin values may indicate a reduced bone turnover during pregnancy, but further studies are needed to clarify this issue and the interaction between sex steroids and GH in the regulation of bone metabolism.

Influence of oral contraceptives on serum levels of angiotensinogen and experimental effects of growth hormone and estrogen (V, VI).

The present ELISA for direct quantification of human angiotensinogen in untreated plasma seemed well suited to measure the series of samples generated during 24-h continuous blood sampling. It is precise, highly sensitive and less time-consuming than other, indirect, methods such as RIA. The mean value in normal
healthy women (n=8) was $1.33 \times 10^{-6} \pm 0.16$ S.E.M. mol/l, range 1.28–1.99x$10^{-6}$ mol/l. The corresponding figure for 18 pregnant women was almost three-fold higher, $3.78 \times 10^{-6} \pm 0.45$ SEM mol/l, range 3.34–6.90x$10^{-6}$ mol/l. These results, as obtained by ELISA, closely correlated with those obtained by RIA ($r=0.97$).

Serum concentrations of angiotensinogen were followed for 24 hours in young women taking oral contraceptives. In all individual profiles a significant elevation in angiotensinogen levels was recorded as compared to pre-treatment values. In fact, all angiotensinogen values measured during treatment were higher than the corresponding values before treatment. The mean values were all 2–4-fold higher during treatment ($p<0.001$). This confirms and extends previous results from studies based on single blood samples (Roberts, 1981). Serum angiotensinogen concentrations are known to increase during pregnancy and in women taking oral contraceptives or estrogens (Gordon, 1983) and hypertension is a well-known complication in these conditions. While serum concentrations of angiotensinogen are regarded as rate-limiting with respect to the renin–angiotensinogen reaction, only a few women with increased angiotensinogen concentrations actually develop hypertension. The exact mechanism of estrogen-induced hypertension is not completely understood. In the present study a careful time-series analysis revealed a marked increase in the variability of serum angiotensinogen levels during oral contraceptive use.

Animal experiments suggest that the increased liver synthesis caused by estrogen administration may be indirect and at least partly mediated via growth hormone.

To study angiotensinogen regulation, experiments were carried out in intact and hypophysectomized male and female rats.

Angiotensinogen concentrations were found to be lower ($p<0.001$) in intact, female (91.7±22.9 AU/l) than in male rats (155.7±5.9 AU/l). After hypophysectomy of female rats, increased ($p<0.01$) concentrations of angiotensinogen (134.9±13.9 AU/l) were recorded. Estrogen substitution after hypophysectomy had no further stimulatory or inhibitory influence. bGH substitution after hypophysectomy differed in its effect, depending on the mode of administration. The increase in angiotensinogen in hypophysectomized female rats was completely reversed after continuous growth hormone administration (85.5±40.0 AU/l). On the other hand when the same dose of bGH (1 mg/kg/day) was given as two daily injections, values (130.0±32.7 AU/l) were in the same range as for hypophysectomized animals receiving no treatment. The importance of an intact pituitary function for seemingly sex steroid dependent liver protein synthesis has been revealed in several studies (Hasegawa et al., 1973; Krakoff & Eisenfeld, 1977), and the sexually dimorphic secretion of growth hormone has emerged as an important mediator of estrogen action (Jansson et al., 1985; Edén et al., 1987). In the present study the rise in angiotensinogen levels caused by hypophysectomy was obviously suppressed by continuous growth hormone administration, whereas the same dose of growth hormone given as two daily injections was ineffective.

Further studies are indicated to explore a possible connection between estrogen induced hypertension and the individual pattern for angiotensinogen variability. A positive correlation between blood pressure and circulating levels of angiotensinogen has been demonstrated in a variety of clinical and experimental
studies (Gordon, 1983). Data suggest that an increased angiotensinogen concentration may be a causal factor in hypertension. Here we found that oral contraceptives not only raise the level of circulating angiotensinogen but also increase its variability. Whether this finding and the individual response profile have any bearing in those few women where hypertension occurs during oral contraceptive use remains to be investigated.

**Interference with biological rhythm.**

An effect mechanism linking environmental factors to metabolic changes was outlined in the introduction. This thesis has shown that during a non-menstrual
state, as exemplified by oral contraception, the secretory pattern of GH is altered. In an experimental study the secretory pattern of GH was shown to affect serum levels of angiotensinogen, a liver-synthesized protein of importance for blood pressure regulation. Angiotensinogen and osteocalcin serum concentrations, used as markers for metabolic changes as regards blood pressure regulation and bone metabolism, were shown to be affected during women's non-menstrual states. Based on the present results, Fig. 16 illustrates a tentative new concept for the mediation of metabolic changes following different exogenous stimuli. In principle, metabolic effects could be mediated either directly via different target organs like bone, blood-vessels, uterus etc. or indirectly via an altered liver metabolism. The present investigation using treatment with exogenous sex steroids as a model suggests a further important mechanism via interaction with central regulatory systems.
CONCLUSIONS

This clinical and experimental study has shown:

• that oral contraceptives alter the secretion of GH towards a pattern of lower and more frequent peaks, but the total amount during 24 h remains unchanged. Previous animal experiments indicate that the secretory pattern of GH is important for a variety of liver metabolic functions.

• that oral contraceptives seem to induce a suppression of the 24-h concentration of CCK, which may be important with respect to weight gain during oral contraception in some women.

• that osteocalcin serum levels display a significant circadian variation where the osteocalcin concentration can vary almost two-fold. This is of great importance when evaluating single blood samples and emphasizes the need for timing of single-point measurements and the value of continuous blood sampling.

• that oral contraceptives may cause a suppression of osteocalcin serum levels with a maintained diurnal variation. The long-term effect as regards future osteoporosis is unknown.

• that during late pregnancy, osteocalcin levels are extremely low and show no diurnal rhythm. This finding could indicate osteoblast inhibition and a decrease in bone turnover.

• that the mode of GH administration is important for the plasma concentration of angiotensinogen in the non-pregnant rat. Estrogen effects on this protein may be mediated via a modification of GH secretion

• that oral contraceptives not only increase angiotensinogen serum levels but also markedly enhance its variability. Further studies are needed to elucidate the relation between the individual pattern of angiotensinogen and hypertension.
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REFERENCES


