CORTICOSTEROIDS IN PLASMA AND SALIVA:

The influence of oral contraceptive use and pregnancy

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STELLINGEN:

1. Bij gezonde vrouwen heeft het gebruik van laag-gedoseerde combinatie-contraceptiva geen hypercortisolisme tot gevolg.

Dit proefschrift.

2. Het gebruik van laag-gedoseerde estrogeenbevattende orale contraceptiva leidt tot een verschuiving in de piek van cortisol naar later in de ochtend.

Dit proefschrift.

3. Het metabolisme van cortisol door 11ß-hydroxysteroiddehydrogenase in speekselklierweefsel resulteert bij de mens niet in corticosteron, maar in cortison.

Clin. Chim. Acta 183:217 (1989).

4. Leeftijdsgebonden osteoporose bij vrouwen kan voor een groot deel voorkomen worden door het levenslang slikken van de "pil".

Voedingsmagazine 7:6 (1994).

5. De foetale leeftijd, waarop een prematuur geborene nog in leven gehouden kan worden, ligt intussen niet ver meer af van het tijdstip, waarop nog legaal een abortus gepleegd mag worden.

Modern Medicine 19:38 (1995).

6. De concentratie van pesticiden in regenwater is bij tijden zo hoog dat, wanneer deze in drink- of oppervlaktewater gevonden zou worden, dit een calamiteit genoemd zou worden.

Chemosphere 28:1559 (1994).

7. Het aantal in Nederland toegelaten bestrijdingsmiddelen is in de jaren 1990-1994 afgenomen. Wanneer vervallen middelen echter worden vervangen door meer potente en/of meer resistente middelen, is het effect op het milieu zeer dubieus.

CBS (1994).

8. De techniek van "molecular imprinting" van antigeenafdrukken in een polymeer zou wel eens het einde kunnen betekenen van het tijdperk van de antilichamen.

Nature 361:645 (1993).

9. Breed-spectrum antilichamen hebben voor een snelle screening in de waterkwaliteitscontrole en de voedingsmiddelencontrole de voorkeur boven zeer specifieke antilichamen. Dit in tegenstelling tot immunoassays voor bijvoorbeeld de klinische diagnostiek.

Biol. Pharm. Bull. 17:843 (1994).

10. De recovery van geaddeerde doelverbindingen kan sterk afwijken van de recovery van dezelfde endogene verbindingen. Bij evaluatiestudies moet hier terdege rekening mee gehouden worden.

Kiwa Symp. nov. (1994).

11. Immunoassays met behulp van een breed-spectrum antilichaam tegen ß-agonisten kunnen goed toegepast worden in de screening op deze verboden groeimiddelen in vlees.

Analyst 119:2671 (1994).

12. ß-Cyclodextrine covalent gekoppeld aan de indicator methylrood reageert op de aanwezigheid van bepaalde organische moleculen met een kleurverandering. Dit maakt dergelijke gastmoleculen geschikt voor de ontwikkeling van een sensor met unieke herkenningsmogelijkheden.

Nature 356:136 (1992).

13. Het is tot nu toe ondanks vele pogingen nog niet mogelijk gebleken een immunosensor voor pesticiden te ontwikkelen.

Rapport RIKZ 94-047.

14. In het kader van het Nederlandse drugsbeleid en uit het oogpunt van economische overwegingen kunnen drugsvangsten het beste gratis of tegen kostprijs verstrekt worden aan verslaafden.

D'eerste trouw

Als van twee gepaarde schelpen
D'ene breekt ofwel verliest;
Niemand zal u konnen helpen
(Hoe of je zoekt of hoe je kiest)
Aan een, die met effen randen
Juist op d'ander passen zou;
D'eerste zijn de beste panden,
Niet en gaat voor d'eerste trouw.

Jacob Cats

Aan mijn ouders en oma

Abstract

Cortisol in saliva is considered to be derived from the free, unbound fraction in plasma by simple diffusion through the salivary gland. Despite considerable conversion into cortisone in the salivary gland by the enzyme 11ß-hydroxysteroid dehydrogenase, levels of cortisol in saliva form a good reflection of the free plasma fraction, which is considered to be the biologically active part. In the present study the relation between plasma free and salivary concentrations of cortisol and cortisone were investigated in women with regard to the influence of hormonal status. Similarly, progesterone was measured in plasma and saliva during pregnancy to demonstrate the relation between plasma free and salivary progesterone. In addition, the daily rhythm of cortisol and cortisone and the influence of hormonal status was investigated on the basis of salivary levels. Further, the effect of processing of salivary samples on the concentration found for several steroids was determined. It appeared that transport of corticosteroids from plasma to saliva is not a simple diffusion proces, but probably proceeds via specific binding proteins in the cell membrane at both the plasma side and the saliva side of the gland cells.

List of Abbrevations

F	= cortisol
E	= cortisone
P	= progesterone
HPAA	= hypothalamo-pituitary-adrenal axis
CRH	= corticotrophin releasing hormone
CBG	= corticosteroid binding globulin
ABG	= aldosterone binding globuline
17OHCS	= 17-hydroxycorticosteroids
UF	= ultrafiltration
ED	= equilibrium dialysis
CBA	= cortisol binding assay
FCI	= free cortisol index
AFCC	= apparent free cortisol concentration
PC	= paper chromatography

CONTENTS

CHAPTER 1

Introduction

1. INTRODUCTION

1.1. The Adrenal

In the human endocrine system the adrenal is a very productive organ. From the more than 40 steroids that have been isolated from the adrenal cortex, cortisol is the main one found in the peripheral circulation. Its secretion amounts up to 25 mg (79 μM) each day. Cortisol belongs to the glucocorticoid hormones. This group of steroid hormones is named glucocorticoids because of their function in the glycogen metabolism in the liver.
Already in 1938 the chemical structure of several adrenal steroids was elucidated [1,2]. At first, determinations of cortisol concentrations were performed in urine with bioassays, being very insensitive and aspecific. In 1952 Nelson [3] described a method to measure adrenal steroids quantitatively in peripheral blood. The measuring of cortisol included purification and the Porter-Silber method [4]. Development of the radioimmunoassay (RIA) increased the reliability, sensitivety and specificity of i.a. cortisol determinations.

Regulation of cortisol synthesis is influenced by the hypothalamo-pituitary-adrenal-axis (HPAA) via a negative feedback system [5,6]. Adrenocorticotrophic hormone (ACTH) secreted by the pituitary induces the adrenal synthesis of cortisol and high concentrations of cortisol in turn inhibit the secretion of ACTH via a reduction of hypothalamic corticotrophin releasing hormone (CRH) secretion. Apart from the negative feedback system the secretion of cortisol is regulated by a circadian rhythm and by stress mechanisms. The circadian rhythm is controlled by an intrinsic "biological clock" mechanism within the midbrain, and is mediated by variation in CRH secretion from the median eminence of the hypothalamus, causing the pituitary to release ACTH. The negative feedback system and the systems controlling the circadian rhythm are clearly interdependent. Independent and much more powerful is the control system which operates during stress. Physical or psychological stress increases the secretion of CRH, ACTH and cortisol, irrespective of the time of day or operation of the feedback [7].

1.2 Cortisol and protein binding

Inherent to its property as a hormone, cortisol is synthesized in an endocrine organ, the adrenal, and transported within the blood to its target tissues. In blood cortisol exists not only in its free form but for the greater part it is bound to proteins, like many other hormones. The discovery of hormone binding, especially steroid binding, was made in the fifties (Forsham 1955 [8], Eik-Nes 1954 [9]) when it appeared that steroids were soluble in plasma to a degree which was supraphysiological. *In-vitro* experiments indicated that albumin,

an abundant plasma protein, bound steroid hormones. By dividing plasma proteins in several fractions, the so-called Crohn's fractions, and performing ultrafiltration or equilibrium dialysis experiments, it could be demonstrated that cortisol in plasma is bound to two proteins, one with high capacity and low affinity, another with low capacity and high affinity [10-[12]). About the same time Daughaday (1958) [12] en Slaunewhite (1959) [13] succeeded in isolating this high affinity binding protein and they named it corticosteroid binding globulin (CBG) and transcortin, respectively. Since that time numerous studies have been performed exploring the nature, properties and significance of CBG, including chemical structure, binding sites, affinities, competition of natural and synthetic steroids, concentrations in several pathological and non-pathological conditions. Summarizing, it appeared that:

1. CBG is an alpha-glycoprotein, with a molecular weight of 52,000 Dalton; it is synthesized predominantly in the liver and it has a half-life in plasma of about 6 days [14].
2. CBG contains one specific binding site for several steroids which show varying binding affinities, the strongest binding being with cortisol [15]. Relative affinities of CBG for steroids, assessed by displacement studies, revealed a descending affinity for cortisol > desoxycortisol > corticosterone > prednisolone > 17-hydroxyprogesterone > desoxycorticosterone > cortisone > progesterone > aldosterone > 11-hydroxyandrostenedione > androstenedione > dehydroepiandrosterone (Mills 1962) [16]. Depending on the method used variations in this order have been found.
3. The distribution of cortisol in blood is about 85% to CBG, 10% to albumin and 5% unbound in normal conditions [17]. These values have been found since the discovery of cortisol and CBG and with minor variations have been stated in several studies [18-20]. A recent survey of the distribution of cortisol between the blood compartments, estimated by computer analysis, is given by Hiramatsu (1987) [21].
4. Concentrations of CBG have been measured in normal and diseased states. These include healthy men and women, children, neonates, fetuses, during pregnancy, during estrogen administration, in Cushing's or Addison's disease, in patients with diabetes, hypogonadism, hirsutism, obesity, hypertension, dysproteinemia, affective disorders and many others. It is beyond the scope of the present study to discuss all these conditions, but a restriction is made to CBG concentrations in normal subjects, during pregnancy and after estrogen adminstration, with a side step to Cushing's disease.

In normal healthy individuals the concentration of CBG is about 30-40 mg/l [22-24]. Pregnancy or estrogen administration leads to a two to three fold increase in CBG levels, accompanied by a concomitant increase in the bound fraction of cortisol [25-28].

The significance of CBG as a high affinity, low capacity cortisol-binding protein has been discussed since its discovery. About that time, 1957, Robbins and Rall [29] who adressed the transport of thyroid hormones, published their free hormone concept, implying that only the non-protein bound fraction of hormones diffuses freely out of capillaries to exert hormonal effects. Several attempts have been made to ascertain that the free hormone concept is also applicable to cortisol. Cortisol as a glucocorticoid hormone is i.a. involved in liver glycogen metabolism. In in vitro and in vivo experiments with mice, Matsui (1966) [30] demonstrated that CBG-bound cortisol is inactive in liver glycogen deposition; cortisol reduction in liver microsomes is reduced in the presence of CBG [31] and the negative

feedback mechanism is correlated with the free fraction of cortisol [31-32].
In man, the best known clinical disorder of supraphysiological cortisol levels is Cushing's syndrome, characterized, the very high cortisol levels and the absence of a diurnal rhythm, as well as obesity, striae, hypertension and other features, which are all considered to be the result of excess of unbound cortisol.

The free hormone concept has been challenged from time to time and a long discussion has been carried on by the groups of Ekins and Partridge [33-38]. Those workers observed and calculated that not only the free but also the albumin-bound fraction of hormones is taken up by organs and that a variable fraction of CBG-bound hormone is taken up by organs like the liver and uterus, depending on the blood capillary transit time relative to the dissociation rate of the hormone from its transport protein. The appearance of CBG in extravascular fluids and the demonstration of specific CBG receptors, however, are some facts that contradict the free hormone concept [16,39].
Mendel's group has also made a considerable contribution to the discussion about the biological activity of free hormones [40-45]. They concluded that the free hormone hypothesis is not likely to be valid for all hormones with respect to all tissues. For cortisol, e.g., it is likely to be valid, but for progesterone it may not hold at all. Nevertheless, the notion of only unbound hormones being biologically active continues to dominate.

1.3. Elevated plasma cortisol

Cortisol determinations are widely used in assessing adrenocortical function. The first methods of measurement developed were rather unspecific, including 1. bioassay, i.e. glycogen deposition in the liver [46]; 2. the amount of 17-hydroxycorticosteroids (17OHCS) in urine [47] and 3. 17OHCS in plasma [42]. 17OHCS were determined fluorometrically [48-51] after extraction of the assay medium and sometimes after some kind of purification.
An interest in adrenal function in pregnancy stimulated Venning (1946) [52] to investigate, with bioassay, the excretion of 17-ketosteroids and corticoids and she found an increase in urinary corticoids in the course of pregnancy. The increase in urinary 17OHCS and a comparable elevation in plasma 17OHCS was later stated [53] and according to Gemzell (1953) [54] the hypertrophic state of the adrenals was a well known phenomenon. The same increase in 17OHCS was found when men with prostatic cancer or postmenopausal women were given estrogens [59,60]
With rather specific methods Cohen (1958) [57] demonstrated that in pregnancy and after estrogen use cortisol and its metabolite cortisone were raised in plasma, but comparable to normal values or even decreased in urine [58-60].

Overall, there were some conflicting data found in the fifties: urinary 17OHCS, particularly cortisol, was considered as a measure of adrenocortial activity, but appeared to contrast increased 17OHCS in plasma during pregnancy and estrogen administration [61-63]. Unlike patients with Cushing's syndrome, pregnant women and women using estrogens showed

no or minimal features of hypercortisolism, despite raised plasma corticosteroids. Differences between these two groups were i.a. the absence of a diurnal rhythm in Cushing's syndrome [64], which was preserved in the pregnancy or estrogen women [62,63], and a difference in CBG levels, which were in the normal range of even slightly decreased in Cushing's syndrome and two to three times higher in pregnancy or after estrogen administration [27].

From that time on the discussion about a state of hypercorticism or hypercortisolism in pregnancy or after estrogen administration was started and continued until recent times.

1.4. Free cortisol measurements

Equilibrium dialysis (ED) or ultrafiltration (UF) are techniques that have since long been used for the separation of molecules of different size. The discovery that cortisol in blood was for the greater part bound to proteins and the discrepancies found between levels of corticosteroids and clinical features of hypercorticism, was the first step towards a long series of attempts to measure the concentration of unbound cortisol in blood or plasma.
The methods applied, besides ED and UF, were ultracentrifugation, gelfiltration and charcoal separation, in recent years also rate dialysis [65]. To acquire physiologically relevant results, the separation of bound and unbound cortisol and subsequently the measurement of one of both fractions, requires that the equilibrium is not disturbed. Many conditions have been described that can lead to biassed results: temperature, dilution of sample, composition of reagentia, pH, leakage of membrane, etc. [27,61-72 a.o.].

The first experiments with regard to unbound corticosteroids were performed with ED and UF by measuring 17OHCS in the sample and in the dialysate [53,73,74]. It is clear that this is a rather unspecific method. The availability of radioactive steroids was an improvement, but despite this the results from several groups were considerably inconsistent, especially when comparing plasma free cortisol levels in normal conditions with those in conditions of elevated CBG. Methodological as well as fysiological factors made comparison of data difficult. The two main factors are: 1. Dilution of the plasma samples; 2. The time of sampling.
Ad.1. Sample dilution disturbs the equilibrium between bound and free fraction of cortisol and this effect is different in plasmas with high or low CBG content. In most studies there was no correction made for the effect of dilution of the samples.
Ad.2. The second main factor is the time of sampling and this is especially important for cortisol which is secreted in a circadian rhythm with peak values in the morning that can be so high that the capacity of CBG is exceeded. As a result of this daily rhythm the concentrations of total bound and free cortisol vary over the day [75].
About 5 % of cortisol in blood is in the free form and equilibrium dialysis is the method of choice to determine the free fraction of cortisol because with a minimal dilution of 1:1 and a thorough control of temperature (37°C), pH (7.4) and buffer composition (isotonic, physiologic) disturbance of the equilibrium will be negligible. The theoretical and practical background of this assertion is described in the dissertation of Ross [76].

1.5 Displacement of cortisol

As described in section 1.2, CBG contains one steroid binding site. Several steroids can bind to CBG with different affinities. The most extended study concerning the association of steroids to the three major plasma proteins in man, SHBG, CBG and albumin, was carried out by Dunn (1981) [77] by measuring the relative binding activity and calculating the association constants for 21 endogenous steroids. With computer simulation and values for total plasma concentrations from literature, for each steroid was determined the % unbound, % SHBG-bound, % CBG-bound and % albumin-bound, in normal men, normal women during both follicular and luteal phase and in women during the third trimester of normal pregnancy.

From the study of Dunn, the results for three steroids are extracted for this study: cortisol, cortisone and progesterone. Besides the interest in cortisol as the basis of this study, cortisone is included because it is the main metabolite of cortisol in plasma and it is elevated in pregnancy. Progesterone is also increased in pregnancy and decreased after the use of oral contraceptives; besides it has strong affinity for CBG. When taking the results of Dunn (1981) it appeared that the relative binding activities were: cortisol = $10 \pm 0.4 \times 10^5$; cortisone = $1.2 \pm \times 10^5$; progesterone = $3.6 \pm 0.5 \times 10^5$. With regard to the parameters calculated, taking into account that plasma values were taken from literature, the distribution of cortisol, cortisone and progesterone was as follows:

Table 1. Distribution of cortisol in blood (from Dunn et al. 1981)

		Total (nM)	% Free bound	% CBG bound	% Albumin
Men	F	400	3.91	89.5	6.57
	E	72	16.2	38.0	45.3
	P	0.57	2.39	17.2	80.1
Women	F	400	3.77	89.7	6.33
(foll.)	E	54	15.8	38.6	44.3
	P	0.65	2.36	17.7	79.3
Women	F	400	3.90	89.4	6.54
(lut.)	E	54	16.0	37.8	44.9
	P	38	2.38	17.2	79.8
Women	F	740	1.82	95.2	2.2
(preg.)	E	110	11.0	59.1	22.0
	P	480	2.28	37.7	54.7

F = cortisol; E = cortisone; P = progesterone; foll. = follicular; lut. = luteal; preg. = pregnant.

In normal men and women cortisol is the major CBG ligand, occupying about 50% of the available CBG-binding sites, followed by cortisone with about 3%. In pregnancy, the decrease of occupancy of CBG by cortisol (about 40%) is primarily the result of competition by the marked increased progesterone (10.6% occupancy), whereas cortisone accounts for 3.8% in this situation.
The competition of cortisol and progesterone for CBG binding sites during pregnancy, when progesterone concentrations are much more increased than those of cortisol had earlier been described in detail by Rosenthal (1969) [78].

1.6. Salivary corticosteroids

In 1957 Robbins and Rall put forward their free hormone concept. The available methods to measure adrenocortical steroid at that time were rather aspecific and it was acknowlegded that errors in the determination of corticosteroids in plasma, total and/or free, could hide physiological significance. In 1955 Killman & Thayson [79] found that the concentration of the drug methylsulphanilamide in human parotid saliva followed closely that of the non-protein-bound drug in venous plasma. Burgen (1956) [80] stated that small non-ionized molecules could freely diffuse through salivary glands. In anticipating that salivary concentrations of synthetic or endogenous hormones might reflect the non-protein-bound fraction in blood, thereby giving a measure of the degree to which they might be available to tissue, a series of experiments was performed to assess adrenocortical activity on the basis of corticosteroids in saliva.
The group of Shannon (1959 a,b, 1960) [81-84] demonstrated that in normal conditions as well as after the administration of cortisol-analogs or ACTH, 17OHCS in parotid saliva parallelled plasma values. Greaves and West (1963) [85] using more specific methods for the measurement of cortisol and cortisone in mixed saliva from pregnant and non-pregnant women, compared their values with plasma unbound cortisol and cortisone values published earlier [86,87]. Their conclusion was that salivary cortisol was much lower than non-protein-bound plasma cortisol, whereas for cortisone the opposite was true. Furthermore, they stated that there is no binding of cortisol and cortisone in saliva and also no enzymatic conversion, suggesting that salivary corticosteroids might reflect plasma free concentrations, but in the case of cortisol oxidation during the transit from plasma to saliva interferes in drawing definite conclusions. It has to be remarked that these authors already mentioned that the method used by them showed considerable error at low levels.

The work of Katz and coworkers was continued leading to another series of publications (1964-1969) [88-96]. Summarizing the results until 1969 it appeared that:
- the main corticosteroids in saliva are cortisol and cortisone;
- in saliva there is no binding of these steroids;
- salivary concentrations parallel plasma dializable concentrations rather than total concentrations;
- salivary cortisol and cortisone exhibit a diurnal rhythm;

- during the transit from plasma to saliva cortisol is converted into cortisone (^{14}C-Cortisol), whereas saliva itself contains no enzymatic activity;
- measurements using Porter-Silber determinations result in a considerable overestimation of corticosteroid levels.

It has to be concluded that although measurement of salivary corticosteroids seemed promising for clinical researchers as a useful alternative to blood to gain more insight in adrenocortical status, it also became clear that the available methods were far from ideal.

As already mentioned by Greaves and West (1963) [85], if hormones in saliva are expected to be a reflection of the non-protein-bound concentration in plasma, the first conditions to be met are that they diffuse freely from plasma to saliva, that they are not metabolized in transit and that there is no protein binding in saliva. Apart from the above mentioned, the methods used for the determination of salivary, plasma total and plasma free concentrations should provide reliable values.

With regard to cortisol in saliva the conversion into cortisone in the salivary gland is most probable, but it is possible that despite this conversion salivary cortisol is still significantly correlated with plasma free cortisol. In that case, salivary cortisol determinations may be useful for assessing adrenocortical activity.

1.7. Survey of literature

In this section a survey of literature data concerning all aspects mentioned above will be given, including the concentrations of cortisol, cortisone, progesterone and CBG measured in plasma and saliva as well as the methods used and also an overview of 11ß-hydroxysteroid dehydrogenase (11ß-HSD). For reasons of clarity those data that were based on unreliable methods or where no sampling time was mentioned or where males were treated with estrogens are omitted, except for results in patients with Cushing's syndrome, because it is known that in that case no diurnal rhythm exists for corticosteroids [61].

1.7.1. Plasma free cortisol

From Table 2 it is clear that in patients with Cushing's syndrome plasma free cortisol is always elevated far above normal values. In pregnancy, although to a lesser degree, plasma free cortisol is also raised in the second and third trimester. The only exception are the results of Jerkunica et al. 1980 [97]. For women taking estrogens the data are varying. Some authors report elevated values, others report values not differing from normal. Remarkable are the findings of O'Connel & Wells (1969) [98] who demonstrated a difference in mean plasma free cortisol in the course of the period of estrogen admininstration. At day 25 mean values were elevated above normal, whereas at day 5 they were not.

1.7.2. Salivary cortisol

With regard to the reported data for salivary cortisol (Table 3) in patients with Cushing's syndrome salivary cortisol was elevated above normal, comparable to plasma free values. For pregnant women the results from the different investigators varied. A significant elevation was only found by Greaves & West (1963) [85] and by Laudat et al. (1987) [132]. For women taking estrogens there are only two studies with reliable results and in these the mean values for salivary cortisol were in the normal range.

1.7.3. Plasma free and salivary cortisone

Plasma free and salivary cortisone have scarcely been measured in a way that comparisons were made between normal women and those women who had taken estrogens or were pregnant, although plasma free cortisone has been determined in cord blood [133]. The only data about salivary cortisone are given by Greaves & West (1963) [84], who stated that in pregnant women salivary cortisone (1.382 ± 0.485 µg/dl, n=31) is elevated above normal values (0.447 ± 0.218 µg/dl, n=20). These values are not totally reliable because for their measurements Greaves & West used fluorimetry and they stated themselves that a variation of about 30% is possible.

1.7.4. Plasma free and salivary progesterone

Regarding plasma free progesterone and salivary progesterone some more data are available. Because it is an important hormone in fertility studies and often necessitates repeated sampling for which saliva has been found to be a good alternative for plasma sampling, salivary progesterone has often been measured during the normal menstrual cycle. Literature data about plasma free and salivary progesterone are shown in Table 4 and 5, respectively.

Table 2: Concentration of plasma free cortisol.

Reference	Group	(n)	Time	Concentration	Remarks
Burke 1969	N-f	31	9.30-10.00	0.66 ± 0.34 µg/dl	Gelfiltration,
[99]	N-m	36	9.30-10.00	0.68 ± 0.47 µg/dl	CBA
	E-f	13	9.30-10.00	1.07 ± 0.37 µg/dl*	
O'Connell & Wells 1969	N-f5	7	9-11.00	1.00 ± 0.15 µg/dl	UF, undiluted
[98]	E-f5	6	9-11.00	1.31 ± 0.12 µg/dl	
	P-3	10	9-11.00	2.47 ± 0.18 µg/dl*	
	N-f25	7	9-11.00	1.09 ± 0.21 µg/dl	
	E-f25	6	9-11.00	1.63 ± 0.07 µg/dl*	
Doe et al. 1969	N-f	10	9.00	1.40 ± 0.40 µg/dl	ED, 1:1.5/1:3
[100]		10	21.00	0.43 ± 0.22 µg/dl	
	E-f	10	9.00	2.31 ± 0.72 µg/dl*	
		10	21.00	0.56 ± 0.18 µg/dl	
	P-3	8	9.00	2.62 ± 0.34 µg/dl*	
		8	21.00	1.02 ± 0.23 µg/dl*	
Burke & Roulet 1970	N-f	31	9.00	0.66 µg/dl	Gel exhange,
[101]		9	24.00	0.18 µg/dl	undiluted
	P-3	15	9.00	1.41 µg/dl*	
		16	24.00	0.64 µg/dl*	
	C	10	9.00	4.03 µg/dl*	
		12	24.00	5.57 µg/dl*	
Lindholm & Schultz-	N-f	14	8-9.00	1.25 (0.42-2.44) µg/dl	UF
Möller 1972			20-22.00	0.23 (0.07-0.86) µg/dl	
[102]	P-3	16	8-9.00	1.84 (0.6-3.4) µg/dl*	
			20-22.00	1.02 (0.1-1.8) µg/dl*	
	E-f	11	8-9.00	2.0 (0.98-2.71) µg/dl*	
			20-22.00	0.60 (0.37-0.86) µg/dl*	

Reference	Group	(n)	Time	Concentration	Remarks
Johnstone & Campbell 1975 [103]	N-f	9	9.00	1.04 ± 0.45 µg/dl	Gel exchange, undiluted
	P-3	10	9.00	1.52 ± 0.40 µg/dl*	
Baumann et al. 1975 [104]	N-f/m	18	8.00	1.38 ± 0.63 µg/dl	ED, undiluted FCI
	E-f	17	8.00	2.04 ± 0.78 µg/dl*	
	P-2/3	12	8-10.00	2.17 ± 0.78 µg/dl*	
	N-f/m	18	17.00	0.46 ± 0.27 µg/dl	
	E-f	17	17.00	0.63 ± 0.32 µg/dl	
Durber & Daly 1976 [105]	N-f	12	9.15-10.00	1.0 ± 0.3 µg/dl	ED, 1:7
	E-f	14	9.15-10.00	2.5 ± 1.1 µg/dl*	
Kley et al. 1977 [106]	N-f	17	8.00	0.94 ± 0.17 µg/dl	ED 1:10, UF
	N-m	18	8.00	0.93 ± 0.14 µg/dl	
	E-f	20	8.00	1.00 ± 0.21 µg/dl	
	P-2	20	8.00	1.37 ± 0.51 µg/dl*	
	C	15	-	3.15 ± 0.86 µg/dl*	
Angeli et al, 1977 [107]	N-f	11	8-9.00	0.84 ± 0.38 µg/dl	Gel exchange, AFCC, Scatchard
	P-3	4	8-9.00	1.47 ± 0.49 µg/dl	
	P-3	10	8-9.00	1.61 ± 0.50 µg/dl*	
Robin et al. 1977 [108]	N-f/m	86	8.00	1.50 ± 0.85 µg/dl	UF
	C	18	8.00	4.40 ± 0.45 µg/dl	
Clerico et al. 1978 [109]	N-f	14	8-9.00	0.87 ± 0.63 µg/dl	ED, 1:5
	N-f/m	32	8.00	0.93 ± 0.45 µg/dl	

Reference	Group	(n)	Time	Concentration	Remarks
Clerico et al. 1979 [110]	N-f/m	40	8.00	0.90 ± 0.46 µg/dl	ED, 1:5
		9	12.00	0.45 ± 0.17 µg/dl	
		10	16.00	0.44 ± 0.17 µg/dl	
		9	20.00	0.28 ± 0.16 µg/dl	
		21	24.00	0.23 ± 0.16 µg/dl	
Matsui et al. 1979 [111]	N-f	29	9.30-11.00	0.4 ± 0.2 µg/dl	ED, 1:1
	P-1	10	9.30-11.00	0.5 ± 0.2 µg/dl*	
	P-2	10	9.30-11.00	0.6 ± 0.2 µg/dl*	
	P-3	9	9.30-11.00	0.7 ± 0.2 µg/dl*	
Predine et al. 1979 [112]	N-f/m	-	8.00	1.42 ± 0.10 µg/dl	UF
	P-3	13	partus	4.60 ± 0.59 µg/dl*	
	C	18	8.00	4.40 ± 0.49 µg/dl*	
Smith et al. 1980 [113]	N	24	8-10.00	1.2 ± 0.4 µg/dl	Charcoal,
		24	16.00	0.4 ± 0.1 µg/dl	1:6, corr.
	E-f	14	8-12.00	1.4 ± 0.5 µg/dl	
	P-1	7	8-12.00	1.2 ± 0.3 µg/dl	
	P-2	6	8-12.00	1.6 ± 0.2 µg/dl*	
	P-3	9	8-12.00	2.4 ± 0.5 µg/dl*	
	C	1	6.1 µg/dl*		
Jerkunika et al. 1980 [97]	N-f	9	8.00	1.10 ± 0.52 µg/dl	UF
			16.00	0.49 ± 0.26 µg/dl	
	P-3	10	8.00	1.26 ± 0.71 µg/dl	
	C	6	-	2.38 ± 0.83 µg/dl*	
Hartmann et al. 1981 [114]	N-f	20	8-9.00	1.46 (1.1-1.9) µg/dl	UF
	P	55	partus	5.63 (3.18-9.96) µg/dl*	

Reference	Group	(n)	Time	Concentration	Remarks
Yamamoto 1982 [115]	N-f	29	9-11.00	0.42 ± 0.14 µg/dl	ED, 1:1
Clerico 1982 [116]	N-f	15	8-10.00	0.67 ± 0.33 µg/dl	ED, AFCC, 1:5
	P-1/2/3	13	8-10.00	1.22 ± 0.26 µg/dl*	
Demey-Ponsart et al. 1982 [117]	N-f	10	18.00	0.32 ± 0.087 µg/dl	ED, 1:10, calculated
	P-3	10	18.00	0.61 ± 0.051 µg/dl	
MacMahon et al. 1983 [118]	N-f	8	8-10.00	1.28 ± 0.62 µg/dl	UF, 1:1.8, corrected
	C	6	-	4.37 ± 1.75 µg/dl*	
Gaspard et al. 1983 [119]	N-f	14	8.30	1.06 ± 0.12 µg/dl	ED, Scatchard (Marvelon)
	E-f	14	8.30	1.42 ± 0.24 µg/dl	
Hiramatsu 1983 [120]	N-m	10	9.00	1.66 ± 0.57 µg/dl	ED, 1:2
	P-3	9	9.00	2.51 ± 0.93 µg/dl*	
Abou-Samra et al. 1984 [121]	N-f	21	9.00	0.40 ± 0.10 µg/dl	ED 1:5
	P-1	16	9.00	0.43 ± 0.12 µg/dl	
	P-2	21	9.00	0.67 ± 0.22 µg/dl*	
	P-3	33	9.00	1.24 ± 0.27 µg/dl*	
Heyns et al 1984 [122]	N-f	5	8.00	3.03 ± 1.60 µg/dl	UF
			16.00	0.72 ± 0.58 µg/dl	
			22.00	0.26 ± 0.13 µg/dl	
	E-f	9	8.00	1.80 ± 0.83 µg/dl	
			16.00	0.49 ± 0.11 µg/dl	
			22.00	0.25 ± 0.05 µg/dl	

Reference	Group	(n)	Time	Concentration	Remarks
Coolens et al. 1987 [123]	N-f	7	8.00	1.65 ± 0.59 µg/dl	UF, calculated
			12.00	0.77 ± 0.66 µg/dl	
			16.00	0.46 ± 0.14 µg/dl	
			22.00	0.30 ± 0.29 µg/dl	
	E-f	11	8.00	1.72 ± 0.77 µg/dl	
			12.00	0.61 ± 0.22 µg/dl	
			16.00	0.47 ± 0.11 µg/dl	
			22.00	0.25 ± 0.05 µg/dl	
	P-1	16	9-17.00	1.05 ± 0.50 µg/dl	
	P-2	18	9-17.00	0.80 ± 0.33 µg/dl	
	P-3	7	9-17.00	1.52 ± 0.37 µg/dl	

Legend: see Table 3.

Table 3: Salivary cortisol determinations.

Reference	Group	(n)	Time	Concentration	Remarks
Greaves & West 1963 [85]	N-f	20	10-12.00	0.11 ± 0.05 µg/dl	PC, Fluor.
	P-3	31	10-12.00	0.244 ± 0.13 µg/dl	
Al-Ansari et al. 1982 [124]	N-m/f	10	8-9.30	0.45 ± 0.18 µg/dl	Kit
			22-23.30	0.11 ± 0.04 µg/dl	
Guechot et al. 1982 [125]	N-m	19	8.00	0.41 ± 0.19 µg/dl	column chrom.
	N-f	45	8.00	0.51 ± 0.26 µg/dl	
	E-f	25	8.00	0.49 ± 0.24 µg/dl	
	P-3	36	8.00	0.51 ± 0.20 µg/dl	
Vining et al 1983 [126]	N-f	5	8.00	0.39 ± 0.10 µg/dl	Direct
			17.00	0.21 ± 0.10 µg/dl	

Reference	Group	(n)	Time	Concentration	Remarks
Silver et al. 1983 [127]	N-m/f	13	9.00	0.20 - 1.03 µg/dl	Kit
			22-23.00	0.02 - 0.17 µg/dl	
Evans et al. 1984 [128]	N-m/f	16	9.00	0.42 ± 0.15 µg/dl	Extract
	N-m/f	10	24.00	0.10 ± 0.06 µg/dl	
	E-f	7	9.00	0.54 ± 0.18 µg/dl	
	P-3	32	9.00	0.38 ± 0.12 µg/dl	
	C	20	9.00	0.90 ± 0.86 µg/dl*	
Deuss et al. 1984 [129]	N-m/f	20	8.00	0.86 ± 0.08 µg/dl	Kit
			9.00	0.71 ± 0.06 µg/dl	
	C	5	8.00	2.36 ± 0.94 µg/dl*	
Luthold et al. 1985 [130]	N-m/f	8	8.00	0.51 ± 0.13 µg/dl	Extract
	C	11	8.00	0.90 ± 0.59 µg/dl*	
Mathian et al. 1985 [131]	N-f	15	8.00	0.76 ± 0.19 µg/dl	Ectract
Laudat et al. 1987 [132]	N-f	35	8.00	0.35 ± 0.019 µg/dl	CBA
		15	20.00	0.11 ± 0.008 µg/dl	
	P-1	14	8.00	0.48 ± 0.068 µg/dl*	
	P-2	13	8.00	0.68 ± 0.088 µg/dl*	
	P-3	12	8.00	0.73 ± 0.081 µg/dl*	
	pp	4	8.00	0.34 ± 0.11 µg/dl	
	C	2	-	1.12 ± 0.22 µg/dl*	

Legend: Group N=normal, m=male, f=female; E=estrogen treated; P=pregnant; pp=postpartum; C=Cushing's syndrome. ED=equilibrium dialysis (dilution of samples); UF=ultrafiltration; CBA=cortisol binding assay; corrected=corrected for one or more parameters; AFCC=apparent free cortisol concentration; Scatchard=calculation with Scatchard analysis. Various methods of saliva measurement are given: PC=paper chromatography; fluor=fluorescence measurement; extract=analysis after extraction; and see Table 3.

Table 4. Plasma free progesterone.

Reference	Group	(n)	Time	Concentration	Remarks
Yannone et al. 1969 [134]	P-W8	1	-	0.08 µg/dl	ED + correction
	W17			0.19	
	W21			0.38	
	W28			0.86	
	W34			0.88	
	W38			0.69	
	W40			1.06	
Greenstein et al. 1977 [135]	P-3	-	-	0.80 µg/dl	Gelfiltration
Anderson et al. 1985 [136]	P-3	12	8-10.00	2.74 ± 0.91 µg/dl	Gelfiltration
Shintani et al. 1987 [137]	N-fol	5	8-12.00	0.006 ± 0.003 µg/dl	
	N-lut	5		0.062 ± 0.010 µg/dl	
	P-1	12		0.203 ± 0.092 µg/dl	
	P-2	10		0.294 ± 0.067 µg/dl	
	P-3	12		0.477 ± 0.134 µg/dl	

Legend: N=normal; fol=follicular; lut=luteal. P=pregnant; W(n)=week (n) of pregnancy; P-1,2,3=trimester of pregnancy.

Table 5. Salivary progesterone.

Reference	Group	(n)	Time	Concentration	Remarks
Walker et al. 1979 [138]	N-lut	9	8-10.00	0.007 - 0.017 µg/dl	Extraction
Connor et al. 1982	N-fol	4	-	<0.005 µg/dl	
[139]	N-lut	4	-	0.040 ± 0.011 µg/dl	
	P-3	3	-	>0.10 µg/dl	
Luisi et al. 1981 [140]	N-lut	9	7-9.00	0.114 ± 0.017 µg/dl	LH-20 column
Zorn et al. 1984	N-fol	32	8-10.00	0.0042 ± 0.0013 µg/dl	Extraction
[141]	N-lut	32		0.0112 ± 0.0005 µg/dl	
Tallon et al. 1984	N-fol	11	7-10.00	0.0016 - 0.0074 µg/dl	Extraction
[142]	N-lut	11		0.010 - 0.027 µg/dl	
	P-3	-	-	0.441 µg/dl	
Webley & Edwards 1985	N-fol	20	-	<0.0022 - 0.0142 µg/dl	Direct
[143]	N-lut	20	-	0.020 - 0.039 µg/dl	
Petsos et al. 1986 [144]	N-lut	6	-	0.0069 - 0.0176 µg/dl	Direct
Bourque et al. 1986	N-fol 14	-		0.0032 ± 0.0018 µg/dl	Direct
[145]	N-lut 14	-		0.0135 ± 0.0075 µg/dl	
Weidenheim et al. 1986	N-fol	8	6-10.00	<0.0047 µg/dl	Extraction
[146]	N-lut	8		0.0248 ± 0.0077 µg/dl	
	P-3	3		0.150 µg/dl	

Legend: see Table 2-4.

The values for plasma free and salivary progesterone show considerable variations, due to the indiviual changes in the course of the menstrual cycle. From Table 4 and 5 it is clear that both plasma free and salivary concentrations in the luteal phase are higher than in the follicular phase and that in the course of pregnancy the values steadily increase.

It must be remarked that the percentage salivary progesterone relative to plasma total progesterone in pregnancy was found to be about 1-2 %, but in the normal menstrual cycle this percentage varied between the follicular and luteal phase from about 9 % to about 2 % respectively [138,140,145]. This difference can be ascribed to the level of CBG, the principal binding protein for progesterone, staying at the same level during the menstrual cycle, but increasing in the course of pregnancy, thereby keeping the percentage of salivary progesterone at the same level.

1.7.5. 11ß-Hydroxy Steroid Dehydrogenase

The existence of the enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD) has been known since the elucidation of the steroid synthesis pathways in the adrenal cortex. Here 11ß-HSD catalyses the last step in the biosynthesis of cortisol from 11-desoxycortisol. Further oxidation of cortisol to cortisone should also be possible in the adrenal cortex but it is generally assumed not to take place, although according to several reports cortisone is present in and secreted by the adrenals [147-149].
11ß-HSD also plays a significant role in the metabolism of cortisol, principally in liver and kidney. The reverse reaction, reduction of cortisone to cortisol, also takes place in the liver.

Cortisone is considered to be a metabolite of cortisol with no biological function. Despite the fact that cortisone has since long been used as a therapeutic agent in, for example, patients with adrenal insufficiency, it is thought that before exerting its therapeutic effect cortisone has to be converted into cortisol. In 1957 Peterson et al. [150] demonstrated that in adrenalectomized rats without liver the administration of cortisone had no effect as life-saving corticosteroid.

11ß-HSD is an enzyme that stands for two activities, oxidase and reductase [151]. Interconversion of cortisol and cortisone has been demonstrated in animals and man by studying the metabolism of radioactively labeled cortisol and cortisone. 11ß-HSD activity is not only present in liver and kidney, but in several peripheral tissues [148,152,153]. A comprehensive overview has been presented by Murphy (1981) [153], summarized in Figure 1.

Figure 1. Distribution of 11β-HSD activities in several human tissues.

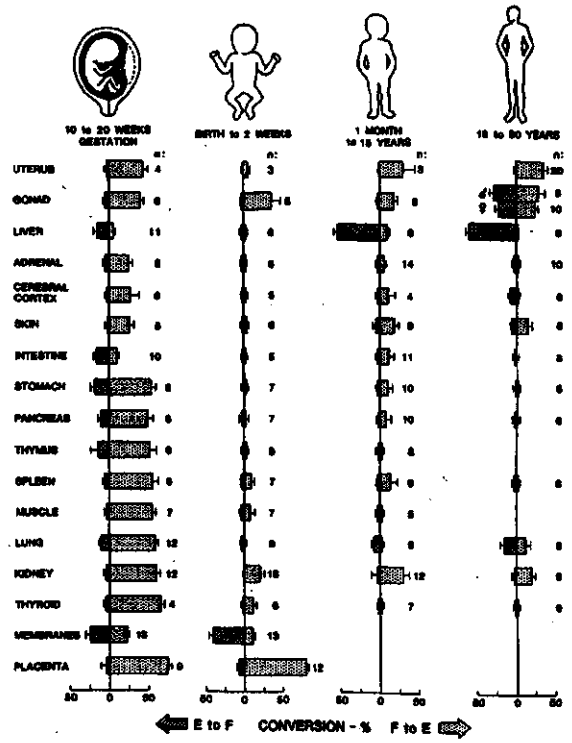

Fig. 1. Interconversion of tracer cortisol (F) and cortisone (E) in various tissues throughout life in the absence of added unlabeled substrate. Conversion from E to F is plotted to the left (stripes) while that from F to E is plotted to the right (dots). Means ± standard errors are indicated.

Adapted from B.E.P. Murphy, J. Steroid Biochem. 14:811-817 (1981) with permission of the editor.

From Figure 1 it appears that in adult man oxidation of cortisol (F) to cortisone (E) predominantly takes place in liver, gonads and lung. Reductive activity was found for the greater part in uterus, gonads, kidney, skin and lung. In contrast to adult man, fetal tissues show a clear predominance of oxidative activity in almost all tissues, being the highest in placenta.

11ß-HSD, at first observed in placenta by Osinsky (1960) [155], has since that time been subject of much study [152-165]. In 1974 Murphy et al. [156] demonstrated that the administration of ^{3}H-cortisol to a pregnant woman resulted in the presence in placenta of almost exclusively ^{3}H-cortisone.
From the results of Tanswell et al. (1977) [158] and Murphy (1977) [152] it appeared that in uterus as well as in placenta the dual action of 11ß-HSD changes from predominantly oxidative to predominantly reductive from the non-pregnant state to the pregnant state until term.

The function of 11ß-HSD in placenta is considered to include the prevention of cortisol reaching the young fetus because cortisol acts as an inhibitor of growth and development of fetal tissues [166]. In the later part of gestation increasingly more cortisol is needed for maturation of the fetal HPAA and lung tissue [167].
Apart from the above mentioned specific placental function and apart from liver metabolism, according to Ferguson & McPhee (1975) [167] 11ß-HSD is associated with tissues involved in water and electrolyte transport. The principal organ in the regulation of water and salt homeostasis is the kidney and 11ß-HSD is present in kidney tissues. The salivary gland is also involved in the excretion of i.a. water and electrolytes. The presence of 11ß-HSD is salivary gland tissue was demonstrated in the dog [90] and the rat [167,168]. In man, salivary gland 11ß-HSD is strongly suggested by the results of salivary cortisol and cortisone measurements compared to plasma (free) values as discussed earlier.
The activity of 11ß-HSD, oxidase as well as reductase, is influenced by several factors. Among these are steroid hormones and there appears to exist a difference in substrate specificity depending on the tissue examined [148,162,163,169-171]. Moreover, some steroids can stimulate the activity of 11ß-HSD, whereas others have an inhibitory effect, again depending on the particular tissue [154,161]. It may be remarked that for example progesterone inhibits the conversion of cortisol into cortisone. Product inhibition by cortisone has also been demonstrated, although in the reverse reaction cortisol shows no product inhibition.

1.7.6. CBG
Since the discovery of CBG in 1958 by Daughaday as the principal high affinity, low capacity binding protein for cortisol and progesterone, this protein has been subject of much investigation. Part of this has already been discussed hereinbefore (see 1.2. Cortisol and protein binding). In the course of time several reviews concerning CBG have been published [15,18,172-175].

The most extensive data about the binding of endogenous steroids (n=21) en drugs (n=70) have been published by Dunn et al (1981) [77] and Pugeat et al. (1981) [176].

1.8. Purpose of the study

Holding to the still generally accepted view that salivary steroid levels are a true reflection of the free, biologically active fraction in plasma, this study was focussed on the relation between cortisol and cortisone in saliva and free in plasma. Regarding the possible biasses in the available methods the assays were optimized. Special attention was given to sampling time.
Because the main interest concerned the influence of a simultaneous increase of CBG and total plasma cortisol on the free plasma-saliva relations, in the first part of the study plasma and saliva samples were collected from a group of women using OC and a group of normally cycling women. In the second part of the study a group of pregnant women was followed from the first trimester until term and they served as their own controls at 6 weeks postpartum.

The following parameters were determined:

- plasma total and free cortisol
- plasma total and free cortisone
- plasma CBG
- salivary cortisol and cortisone.

In the pregnancy group the number of parameters was extended with:

- plasma total and free progesterone
- salivary progesterone.

The main purpose of the study was to gain more information about the following issues:

1. Which (cor)relations exist between salivary and plasma concentrations of cortisol and cortisone in the three study groups?
2. Will, after OC usage or during pregnancy, the simultaneous increase in plasma total cortisol and CBG lead to a hypercorticism at the level of plasma free cortisol and is this reflected in saliva?
3. What is the influence of 11ßHSD on salivary cortisol and cortisone levels?
4. Is salivary progesterone a reflection of plasma total and/or free progesterone?
5. To what extent has the influence of the daily rhythm to be taken into account?

1.9. References

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CHAPTER 2

The effect of oral contraceptives on plasma-free and salivary cortisol and cortisone

The effect of oral contraceptives on plasma-free and salivary cortisol and cortisone

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Summary

The effect of a low estrogen oral contraceptive (OC) on glucocorticoid levels in plasma and saliva as well as glucocorticoid binding was studied in 23 healthy women using 30 µg ethinyl estradiol (EE2) + 150 µg desogestrel (Marvelon®) (II).
Fifteen healthy females with normal menses served as controls (I). Blood and salivary samples were taken between 9.00 and 9.30 a.m. on the 18th day of menstrual or pill cycle. Assay accuracy had been optimised by applying extraction and chromatographic purification before radioimmunoassay (RIA) of cortisol and cortisone in both plasma and salivary samples. Free steroid assays were performed by applying the same procedure to equilibrium dialysates obtained after dialysing plasma against an equal volume of buffer, instead of measuring tracer distribution. Corticosteroid Binding Globulin (CBG) was measured by a commercial RIA. As expected, CBG as well as plasma total cortisol were elevated in the pill group. Interestingly both plasma free and salivary cortisol were higher than in controls (free cortisol I: 18.0 ± 7.95 nmol/l; II: 32.3 ± 9.03 nmol/l; salivary cortisol I: 9.2 ± 3.88 nmol/l; II: 18.8 ±6.92 nmol/l. Salivary cortisol closely parallelled plasma free cortisol both within and between the groups, though at a much lower level (about 50%). Free cortisone was slightly lower in the pill group (I: 10.8 ± 2.55 nmol/l; II 8.5 ± 1.86 nmol/l) whereas salivary cortisone was 2.3 (I) and 4.4 (II) times higher than plasma free cortisone and tended to follow the plasma free and salivary cortisol pattern, both within and between the study groups. These latter observations were taken as suggestive of the presence of 11ß-hydroxysteroid dehydrogenase (11ßHSD) in the salivary gland, converting part of the entering cortisol into cortisone. Nevertheless, salivary cortisol must be considered to reflect adequately the increased plasma free cortisol during OC-administration.

Introduction

The estrogen-induced rise in plasma cortisol and its principal carrier protein CBG has been known for a long time [1,2]. However studies on plasma free cortisol levels during OC-use have hitherto led to contradictory results [3-20]. The large variety in the reported observations can at least partly be ascribed to assay methodology. Furthermore, the diurnal rhythm in cortisol secretion makes sampling time a critical factor.
Most of the steroid hormones in plasma have been detected in saliva as well. Salivary levels of the unconjugated steroids are usually in the same order of magnitude as their correspon-

ding free concentrations in plasma [21,22]. This suggests that salivary steroid hormones derive directly from the plasma free moiety. Obvious advantages of saliva as a medium for hormone assay are its ease of accessibility and the stress-free conditions for obtaining samples. Also, methodological problems involved in free hormone assay can be circumvented. The few literature date available [23,24] indicate that salivary cortisol remains within the normal range during OC use. Furthermore, it has been demonstrated that salivary levels of cortisol are substantially lower than plasma free levels [25,26], possibly due to metabolic conversion of cortisol to cortisone by an 11-hydroxy steroid dehydrogenase (11ß-HSD) in the salivary gland [6,27].
This report presents the results of the measurements of plasma cortisol and cortisone, both total and free, and their respective salivary levels in a group of healthy women using an OC combination of ethinyl-estradiol and desogestrel, and in healthy controls with normal menses. To minimize methodological artefacts, free cortisol and cortisone were assessed by equilibrium dialysis of plasma against an equal volume of buffer, followed by highly specific RIAs including extraction and chromatographic purification of the free hormone in the dialysate. The same RIAs were employed for total and salivary steroids.

Materials and methods

Subjects

All subjects cooperating in this study were healthy female volunteers, aged 18-45 yr with a history of regular menses. The control group (I) consisted of 15 women with a normal menstrual cycle. The OC group (II) included 23 women taking ethinyl estradiol 30 µg + desogestrel 150 µg (Marvelon®). The subjects in group II had been using the same preparation for at least 6 mth.

Sampling

Matched samples of blood and saliva were taken at 9.00-9.30 h a.m. at day 18 of the menstrual or pill cycle. Blood was drawn into heparinized tubes, centrifuged and the plasma stored at -20°C until analysis. Citric acid stimulated saliva was collected in plastic cups, frozen at -20°C and sonicated after thawing.

CBG

Plasma CBG concentrations were determined with a CBG RIA kit purchased from Medgenix, Brussels, Belgium.

Plasma total and salivary cortisol and cortisone

For the determination of total plasma and salivary cortisol and cortisone 0.2 ml of plasma or 0.7 ml of saliva were incubated with ±10.000 dpm of recovery tracer (1,2,6,7-^{3}H-cortisol 83.5 Ci/mmol and 1,2-^{3}H-cortisone 29 Ci/mmol) for 30 minutes. The samples were extracted with 14 ml of dichloromethane and the extracts dried under a stream of clean air. Residues were applied on Whatman no. 1. paper and chromatographed in a descending Bush B5 solvent system (toluene:methanol:water 2:1:1). After radioactivity scanning, the

peaks were eluted in 2 ml ethylene glycol in water (EGW) and the eluated used in the RIA and for recovery counting. Standard curves were set up in duplicate. After overnight incubation at 4°C the bound and free fraction were separated with dextran-coated charcoal.

Free plasma cortisol and cortisone

For the determination of the free plasma concentration of cortisol and cortisone 0.8 ml of plasma was dialysed against 0.8 ml buffer (0.05 mol/l phosphate, 0.1 mol/1 NaCl, pH 7.40) for 3 h in a Diachema dialysis apparatus. The concentrations of the hormones were measured in the dialysate using the same procedure as described for total plasma and salivary cortisol and cortisone. The free fractions fo' and the free hormone concentrations Fo' were estimated for undiluted plasma using a formula that takes into account the dilution (1:1) implicit in equilibrium dialysis:

$$f_o' = 1/(H_o/F\text{-}V_f/V_o+1)$$

$$F_o' = H_o \text{ X } f_o,$$

where: Ho = total hormone concentration (nmol/l; F = (free) hormone concentration as measured in dialysate; Vf/Vo = ratio of total volume of dialysis system to sample volume, which for the present case equals 2.

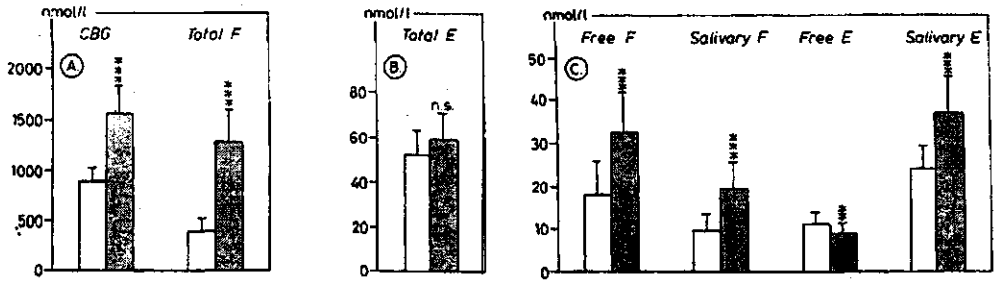

*Fig. 1. Concentrations of CBG, cortisol (F) and cortisone (E) in plasma and saliva in group I (controls) and group II (pill). Mean values ±sd: CBG: I 892 ± 123 nmol/l; II 1534 ± 284 nmol/l; plasma total F: I 394 ±394 ± 107 nmol/l; II 1284 ± 315 nmol/l; plasma total E: I 52 ± 10.4 nmol/l; II 59 ± 10.6 nmol/l; plasma free F: I 18.1 ± 7.95 nmol/l; II 32.3 ± 9.03 nmol/l; plasma fee E: I 10.8 ± 2.56 nmol/l; II 8.5 ± 1.86 nmol/l; salivary F: I 9.2 ± 3.88 nmol/l; II 18.8 ± 6.92 nmol/l; salivary E: I 24.2 ± 5.43; II 36.7 ± 8.45. Significance levels (t-test): *** p<0.0001; ** p<0.01; n.s., not signifant.*

Results

The results of determinations of the CBG, total and free plasma as well as salivary cortisol and cortisone concentrations are shown in Fig. 1. In the OC group, CBG and total plasma cortisol were significantly elevated with respect to the control group. Although elevated CBG leads to a substantial reduction of the percent free cortisol, the mean free cortisol concentration was significantly higher than the mean of control values. Salivary cortisol levels parallelled plasma free levels, both within and between groups (Table I). The absolute levels of salivary cortisol, however, were lower than plasma free cortisol by a factor 2.

Table 1.
Comparison of plasma free and salivary cortisol (F) and cortisone (E) in the control (I) and the pill (II) group.

	I (controls) (n=15)	II (EE2+desogestrel) (n=23)	t-test
FF(nmol/l)	18.1 ±7.95	32.3 ±9.03	a
SF(nmol/l)	9.2 ±3.88	18.8 ±6.92	a
Ration SF/FF	0.52 ±0.11	0.58 ±0.12	n.s.
Corr. SF/FF	0.92 [b]	0.81 [b]	
FE(nmol/l)	10.8 ±2.56	8.5 ±1.86	c
SE(nmol/l)	24.2 ±5.43	36.7 ±8.45	a
Ration SE/FE	2.29 ±0.44	4.44 ±1.03	a
Corr. SE/FE	0.66 [c]	0.48 [d]	

FF, plasma free cortisol; SF, salivary cortisol; FE, plasma free cortisone; SE, salivary cortisone; significance levels: [a]= p<0.0001; [b]= p<0.001; [c]= p< 0.01; [d] = p<0.05; corr, correlation coefficient (Pearson); n.s = not significant.

Plasma free cortisone was slightly but significantly reduced ($p<0.01$) as compared to controls. Salivary cortisone exceeded free cortisone considerably. A significant correlation between the two parameters existed within groups (Table I), but their ratio differed appreciable between groups. Salivary cortisone concentrations were more or less similar to plasma free cortisol values. Moreover, the within group correlations between salivary cortisone and free cortisol were as good as or even better than between salivary and free cortisone ($r_1 = 0.64$, $r_2 = 0.76$), and taking the two groups together, the correlation between salivary cortisone and free cortisol remains highly significant ($r = 0.84$, $p < 0.001$), whereas the correlation with plasma free cortisone disappears ($r = 0.04$, n.s.). These findings suggest that plasma free cortisol contributes significantly to salivary cortisone.

Discussion

The well-known effect of estrogen administration on hepatic CBG synthesis, its plasma level and the concomitant rise in total cortisol is apparent also with low dose estrogens. According to the present results, both plasma free and salivary cortisol are elevated too. Others [7-10,13] have reported elevated free cortisol levels, but unmodified free cortisol during OC use also has been found [19]. By the application of equilibrium dialysis at the lowest dilution that is technically feasible, using a buffer with physiological pH, ionic strength and chloride concentration, and employing a highly specific RIA with chromatographic prepurification for free hormone determination in the dialysate, not much room seems to be left for doubting the validity of the assay results. The observation seems however to conflict with the free hormone concept [28], which implies that the free hormone concentration reflects its bioavailability. This would raise the question why no overt clinical signs of hypercorticism are observed. As it is uncertain whether this observation, which is done between 9.00 and 9.30 a.m., is representative of the average daily exposure of the body to glucocorticoid action, the elevated free and salivary cortisol may merely reflect a shift of the morning peak of cortisol. Alternatively the observation would be consistent with anti-glucocorticoid action of one of the components of the OC preparation, both at the pituitary and target organ level.

A relatively tight relation exists between salivary and plasma free cortisol (Table I). Correlation is highly significant within both control and OC groups, and the mean ratio salivary/free cortisol is almost the same for control and OC subjects. This explains why the correlation further improves when all observations are taken together. Remarkably, salivary cortisol is only 50% of its plasma free counterpart, but nevertheless may be considered as representative of free cortisol in OC users and non-users by virtue of the above mentioned relations. This is in line with other observations [22,26], showing that salivary cortisol closely parallels the pattern of free cortisol. The relation between salivary and plasma-free cortisone appears to be much less unambiguous. Within both groups, a significant correlation exists, which however disappears when the two groups are taken together. This is because the ratios of salivary to free cortisone differ widely for controls (2.3) and subjects on OC (4.4). The fact that these ratios are well over unity in both cases suggests the presence of an additional source of salivary cortisone. Remarkably, there is a significant correlation between salivary cortisone and plasma free cortisol which is as good as or even better than with free cortisone, and which does not disappear when combining the two groups, but rather improves. These findings, together, with the indications of the presence of 11ß-HSD in the salivary gland [6,27], led us to hypothesize that salivary cortisone originates for one part from plasma free cortisone and for the other part from conversion of cortisol into cortisone in the salivary gland. This would also account for the fact that apparently some cortisol is 'lost' at the transition from plasma to saliva.

In summary the following conclusions are drawn. During EE2/desogestrel use, both plasma free and salivary cortisol increase appreciably. A ratio of approximately 2:1 between these two parameters exists normally and remains in the presence of high CBG levels induced by the OC combination administrated. Therefore, salivary cortisol may be considered as an adequate parameter reflecting plasma free cortisol. This does not count for salivary cortiso-

ne. It exceeds free cortisone by a factor that is strongly related to free and salivary cortisol. The observations are consistent with the presence of cortisol-to-cortisone converting activity.

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CHAPTER 3

Salivary progesterone excellently reflects free and total progesterone in plasma during pregnancy

Salivary progesterone excellently reflects free and total progesterone in plasma during pregnancy

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ABSTRACT

To see if saliva is a valid substitute for plasma in assay of progesterone even when concentrations of hormone and binding proteins are fluctuating, we determined the concentrations of total and free progestrone in plasma and salivary progesterone from 36 women volunteers during the course of pregnancy and six weeks postpartum, using a highly specific RIA after extraction and chromatographic purification of the steroid. The free fraction in plasma was determined via equilibrium dialysis, followed by the same RIA analysis for progesterone in the dialysate. Desspite the dramatic increases in concentrations of total progesterone and binding proteins in plasma during pregnancy, we found highly significant correlations between total and free progesterone in plasma and salivary progesterone in the group as a whole as well as individuals ($P < 0.001$ in almost all cases). The proportion of free progesterone in plasma and of salivary progesterone relative to total progesterone in plasma remained constant at about 1 % and 0.5 %, respectively, whereas during the postpartum period there was much more variance. Evidently salivary progesterone is a very good alternative to plasma as a sample for use in follow-up during pregnancy.

INTRODUCTION

Since the discovery that steroid hormones are present and measurable in saliva (1), the clinical advantages of using this kind of sample for their determination have been described several times (2-5). Concentrations of unconjugated steroids are quantitatively similar to the free fractions in plasma. Total and free concentrations in plasma and in saliva correlate well. The free moiety of steroid hormones in plasma relates to their biological activity (for review, see reference 6). For these reasons the use of saliva has clinical as well as scientific interests, especially in situations characterized by altered concentrations of binding proteins.

During pregnancy, concentrations of steroids and steroid binding proteins in blood are rapidly changing, making samples of pregnant women eminently suitable for the study of correlations between saliva and plasma steroids over a wide range of values. We were especially interested in progesterone, a hormone synthesized by the adrenals and gonads and, during pregnancy, by the placenta. Its total concentration in plasma increases from <10 μg/L in the follicular phase to about 170 μg/L at term (7-10).

During the menstrual cycle, when changes in progesterone concentrations are less dramatic than during pregnancy, salivary progesterone gives a good reflection of progesterone con-

centrations in plasma, although authors disagree about the actual concentrations in saliva and about the correlations between salivary and plasma concentrations of progesterone (11-13). During pregnancy, again, the enormous increase in plasma progesterone is reflected in saliva (14,15). Unfortunately, few studies have dealt with the relation between total and free progesterone in plasma and that in saliva throughout gestation. Darne et al.(15) demonstrated an excellent correlation between free progesterone in plasma and salivary progesterone in the second half of pregnancy, but contradictory results were published by Perry et al.(9).

In our study we assayed matched blood and saliva samples from 36 pregnant women during gestation and postpartum. Total progesterone in plasma and salivary progesterone were determined with a highly specific and sensitive RIA, including chromatographic purification before assay. Free progesterone in plasma was determined by equilibrium dialysis, followed by RIA of the dialysate.

Materials and methods

Subjects and sampling

Thirty six healthy pregnant women, attending the Gynecologic Clinic of the St.Jozef Hospital in Doetinchem for obstetrical reasons, volunteered in this study.

Matched blood and saliva were sampled four times during pregnancy - during weeks 14-19 (period 1), weeks 20-26 (period 2), weeks 27-34 (period 3), and weeks 35-40 (period 4) - and once at six weeks postpartum (period 5). From half of the women we collected blood and saliva specimens at 0900-1000 h, and from the other half at 1500-1700 h. Blood was drawn into heparinized tubes, centrifuged (5 min at 1500 x g), and the plasma was stored at -20 °C. Saliva was collected in plastic cups and frozen at -20 °C. Before use it was thawed, centrifuged (10 min at 1500 x g), and the supernatant liquid was used for the analysis.

Methods

For the assay of total progesterone in plasma and salivary progesterone we incubated 0.2 and 2.0 mL samples, respectively, with recovery tracer, about 10 000 dpm [1,2,6,7-^{3}H]-progesterone (4.07 PBq/mol, New England Nuclear Corp., Boston, MA), for at least 2 h. We then extracted the samples with 15 ml of diethyl ether. The residues were applied to Whatmann no.1 paper strips and chromatographed in a descending Bush B3 solvent system [petroleum ether (b.p. 80-110°)/toluene/methanol/water, 133/167/400/100, by vol]. All chromatography solvents were of "Baker Analyzed" grade (J.T.Baker Chemical Co., Phillipsburg, NJ). After chromatography was completed we scanned of the paperstrips for radioactivity and eluted the progesterone peaks ($Rf \approx 0.88$) in 2.0 mL of borate buffer (0.1 mol/L, pH 8.0) containing 5 g bovine serum albumine per liter. The eluates were used for assessing analytical recovery and RIA.

In the RIA three different volumes of eluate were incubated with [^{3}H]-progesterone and antiserum (raised against progesterone-11-hemisuccinate-BSA, a gift from Organon, Oss, The Netherlands). After incubation for at least 2 h at 4 °C the bound and free fractions were separated by use of dextran-coated charcoal. The detection limit of the RIA (3 SD from the

mean of the zero standard, n = 10, was 1.5 pg/tube. The procedural recovery was about 50 %, resulting in a detection limit of 90 ng/L for total progesterone in plasma and 10 ng/L for salivary progesterone. Interassay CV was 8.4 % for progesterone concentrations of 1 µg/L (n=48). The intra-assay CV for total progesterone in plasma at 125 µg/L was 8.1 % (n=10). The intra-assay CV for salivary progesterone at 0.6 µg/L was 6.7 % (n=10).
The free fraction of plasma progesterone was determined by equilibrium dialysis according to the method of Meulenberg et al.(16), briefly described as follows. We dialyzed 0.8 mL of plasma against 0.8 mL of sodium phosphate buffer (50 mmol/L, pH 7.40) for 3 h at 37 °C. After dialysis the concentration of progesterone in the dialysate was determined by the same procedure as for total progesterone in plasma. After correction for sample dilution (1:1) in the equilibrium dialysis, we calculated the free progesterone concentration in plasma.
For statistical analysis of the results we used Student's t-test and Pearson regression analysis.

Results

Figure 1 illustrates the courses of the concentrations of progesterone in plasma and saliva during pregnancy and six weeks postpartum. Because half of the samples were collected at 0900-1000 h and the remaining half at 1500-1700 h, we have separated the values for morning and afternoon. Shown are the mean concentrations of progesterone at periods 1 to 5.
In the samples collected in the morning, mean total progesterone concentration increased from 48 µg/L in period 1 to 170 µg/L in period 4, then sharply declined to 1.5 µg/L in period 5. The mean concentrations in the afternoon samples showed the same course: an increase from 41 µg/L to 180 µg/L and then a decrease to 4.4 µg/L. We detected no statistically significant differences between morning and afternoon samples (Student t-test) (Figure 1a).
Free progesterone in plasma increased from 0.6 µg/L to 2.2 µg/L from period 1 to 4, then decreased to 0.12 µg/L postpartum in the morning samples. In the afternoon samples the mean concentrations were 0.47, 1.75 and 0.09 µg/L, respectively. Plasma free progesterone concentrations in morning and afternoon samples were significantly different ($P < 0.05$ by Student's t-test) during pregnancy, with higher values in the morning (Figure 1b).
The mean concentration of progesterone in the corresponding saliva samples, although about 50 % lower in absolute values, showed the same pattern as for plasma free progesterone. It increaes from about 0.20 µg/L in period 1 to about 0.90 µg/L in period 4, and then declined to a mean of 0.03 µg/L at six weeks postpartum (Figure 1c). There was no statistically significant difference between samples collected in the morning and afternoon.
Most of the progesterone in plasma is bound to proteins (corticosteroid binding globulin, albumin), only 1-2 % being in the free form. We found that during pregnancy (periods 1 to 4), the percentage of free progesterone relative to total progesterone in plasma ($\approx$ 1 %) varied little, even though the latter increased to about 100 times postpartum values. Postpartum, the mean proportion of free progesterone in plasma was 10-17 % (Figure 2a). Progesterone in the corresponding saliva, expressed as the mean percent of the total progesterone in plasma, was also more or less constant during pregnancy, varying from 0.45 %

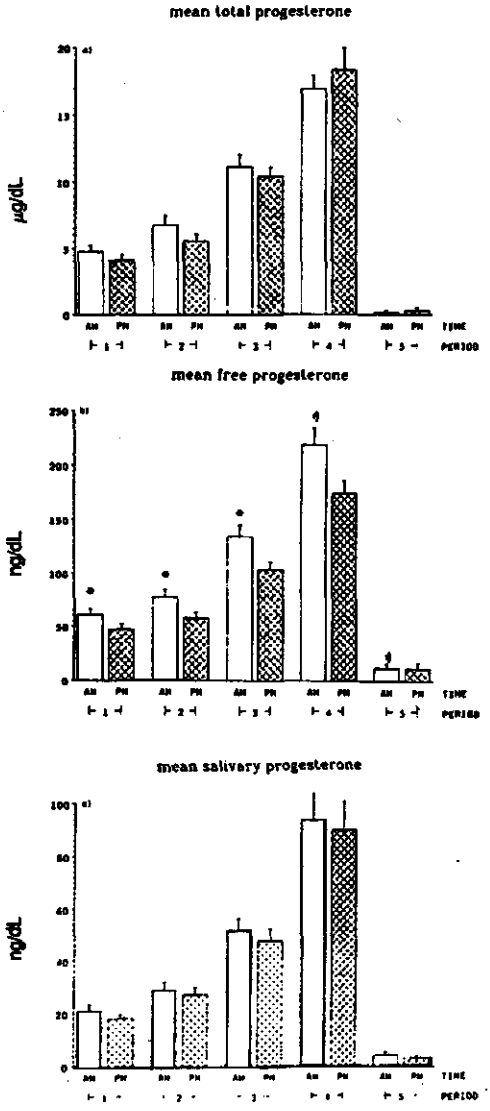


Figure 1.
The mean (± SEM) concentrations of total (a), and free (b) progesterone in plasma, and salivary (c) progesterone during pregnancy and at six weeks postpartum.
** P < 0.05 Student's t-test, difference between morning (AM) and afternoon (PM) samples; 1) not significant.*

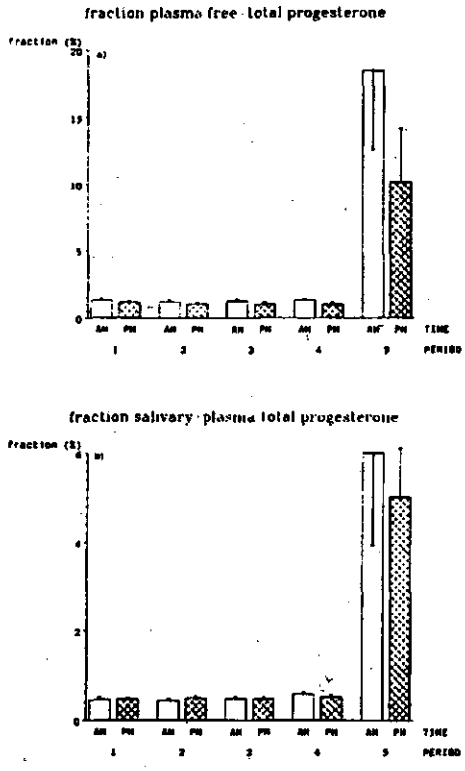
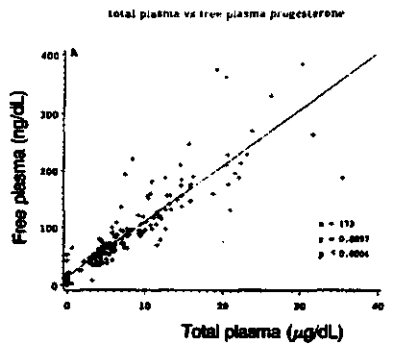
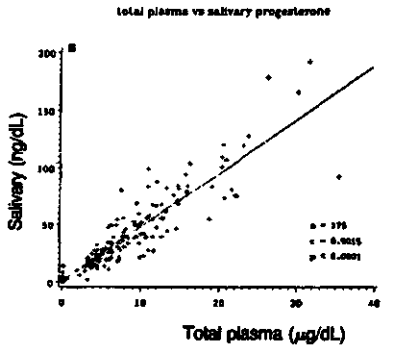


Figure 2.
The mean (± SEM) percentages of (a) free progesterone in plasma and (b) salivary progesterone relative to total progesterone in plasma.





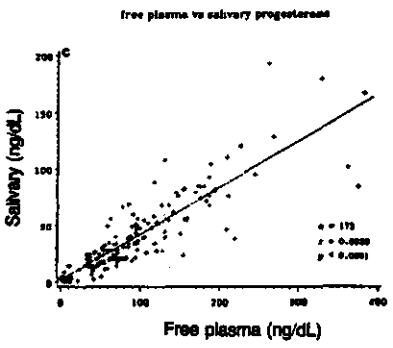
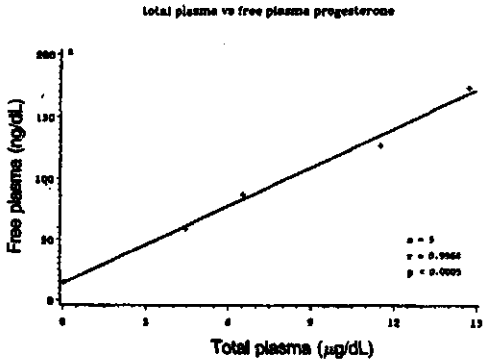
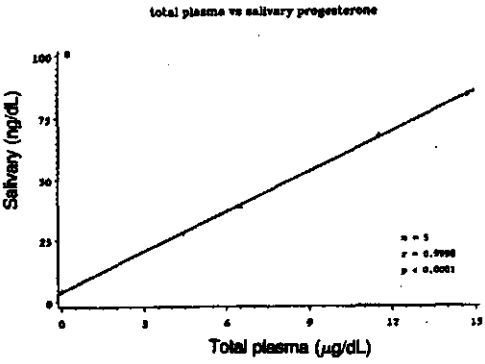


Figure 3.
Correlations between total and free progesterone in plasma and salivary progesterone.





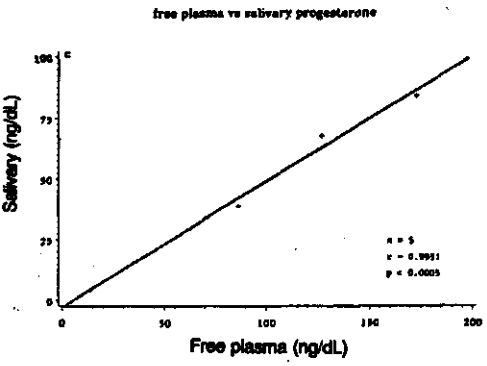


Figure 4.
Correlation between total and free progesterone in plasma and salivary progesterone for one subject.

to 0.56 %. Postpartum, it was 5-6 % (Figure 2b).
The correlations among the three parameters (Figure 3) were highly significant Table 1). Despite the small number of observations per person, correlation coefficients and P-values for individual subjects were almost without exception highly significant. An example is given in Figure 4.

Table 1
Comparison of total and free progesterone in plasma and salivary progesterone.

	Variable	Line	r	n	p	SD(y)
A	TP vs FP	y = 0.010 x + 0.133	0.8897	173	0.0001	0.0004
	TP vs SP	y = 0.005 x + 0.021	0.9015	175	0.0001	0.0002
	FP vs SP	y = 0.408 x + 0.013	0.8828	173	0.0001	0.0168
B.	TP vs FP	y = 0.011 x + 0.135	0.9964	5	0.0005	0.0005
	TP vs SP	y = 0.005 x + 0.038	0.9998	5	0.0001	0.0001
	FP vs SP	y = 0.516 x - 0.028	0.9951	5	0.0005	0.0298

TP = Total progesterone in plasma, FP = free progesterone in plasma, SP = salivary progesterone; all values are in µg/L.
r = correlation coefficient (Pearson), n = number of subjects, P = significance level, SD(y) = standard deviation of y; A, all samples; B, one subject.

Discussion

In agreement with many former studies, our results illustrate the dramatic increase in total progesterone in plasma during pregnancy, with mean maximum values of about 170 µg/L at term. We found a similar increase for free progesterone in plasma and salivary progesterone (mean maximal values of about 2.0 µg/L and 0.90 µg/L, respectively) (Figure 1). The three variables werd highly significant correlated ($P < 0.001$) for the study group as a whole as well as for individual subjects. Thus we conclude that salivary progesterone gives a very good reflection of total as well as of free progesterone in plasma during gestation.
Although the proportions of free progesterone in plasma and salivary progesterone relative to total concentrations in plasma remained constant during pregnancy at about 1 % and 0.5 %, respectively, we found much higher proportions, with considerable variation, post partum (Figure 2). Considerable variations in the ratio of salivary progesterone to plasma total progesterone during the menstrual cycle have been reported by others (10,17). Our finding that mean free concentrations in plasma were significantly higher than salivary concentrations is in line with data presented by Darne et al.(15). The fact that steroid-metabolizing enzymes have been found to be present in salivary glands (16,18-20) and gingival tissue

(21) may provide an explanation for this phenomenon. A second explanation for the discrepancy may be the loss of progesterone during centrifugation of the saliva before analysis. From the study of Lequin et al.(22) it appeared that the centrifuged cell debris of saliva contained disproportionately high amounts of progesterone. Nevertheless, diminished concentrations in saliva apparently do not disturbe the correlation between plasma and saliva.

We divided our study group in a morning and an afternoon group with respect to sample collection. The respective results for these two groups (Figure 1) show that only for free progesterone in plasma was there a statistically significant difference between the means for morning and afternoon samples during periods 1 to 4, with higher values in the morning. A possible explanation for this finding lies in the daily rhythm of cortisol and the competition of progesterone and cortisol for their combined binding protein, the corticosteroid-binding globulin. The two steroids bind to the latter at the same place and with almost the same affinity (23,24). During the morning peak of cortisol, progesterone will be displaced from corticosteroid-binding globulin, resulting in an increased proportion of free progesterone (8,25). We did not find this daily variation to be reflected in progesterone concentrations in saliva. This finding can be explained with the assumption that the competition between cortisol and progesterone continues during the passage from blood to saliva via the salivary gland. Accordingly, we tend to believe that the transport of steroids is not merely a process of passive filtration, but rather an active process, a view already postulated by Lequin et al.(22).

In conclusion, it is clear that during pregnancy the increase in total progesterone concentrations in plasma is reflected both in the proportion that is free in plasma and in the concentrations in saliva, with highly significant correlations among these three parameters for our study group as a whole as well as for individual subjects. Consequently, saliva is an excellent alternative to plasma for the analysis of progesterone for follow-up during pregnancy.

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CHAPTER 4

The effect of pretreatment of saliva on steroid hormone concentrations

The effect of pretreatment of saliva on steroid hormone concentrations

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SUMMARY

We investigated the effect of the pretreatment (sonification or centrifugation) of saliva samples on the concentration of several steroid hormones as measured with highly specific RIA after extraction and chromatography. It appeared that sonification of saliva resulted in significantly higher values for progesterone, cortisone, 17-hydroxyprogesterone, testosterone and estradiol (10 - 49 % increase), compared with the levels recorded after centrifugation of the samples. No differences were demonstrated for the concentrations of cortisol and androstenedione, except that a sex-dependent difference was observed in the values for androstenedione: concentrations measured in sonificated male saliva were lower than those measured in supernatant saliva.

INTRODUCTION

Hitherto, the rationale of steroid assays in saliva was based on several assumptions:
1. that the plasma free, protein-unbound, apolar steroid hormones freely diffuse through the salivary gland,
2. that the concentrations of these steroids in saliva are comparable to their plasma free levels,
3. that steroid binding proteins are absent from saliva (1,2).

Indeed, many authors report a very close relationship between the plasma free and salivary concentrations of several endogenous steroids, synthetic steroids and even drugs (3-9). The very fast (infusion-studies) and flow rate-independent appearance of apolar steroids in saliva strengthened the idea of an unhampered diffusion from plasma to saliva (10). In those cases where salivary concentrations did not reflect their plasma free levels, or where plasma free and salivary concentrations were quite different, this was explained by assuming the presence of steroid metabolizing enzyme activities in the salivary gland or by methodological imperfection (11-15).

However, it was later demonstrated that steroids in saliva exist only partly in the unbound state (4,16-20). The assumption that only apolar, lipid-soluble or small polar compounds could enter the saliva by diffusion or ultrafiltration, respectively, had to be reconsidered when several proteins were detected in saliva (21-28). The most disturbing finding, which may result in a re-evaluation of the current concepts in salivary steroid assay, is the detec-

tion of specific steroid binding proteins in saliva: corticosteroid binding globulin, sex hormone binding globulin, aldosterone binding globulin (29-32). According to Chu & Ekins (1989), corticosteroid binding globulin in saliva, which is highly dependent on flow-rate, is not merely a contamination, but should be regarded as a secretion product, originating from plasma.

The generally applied technique of saliva preparation before assay is freezing, thawing and centrifugation, which produces a clear, easily pipettable supernatant. Freezing and thawing of saliva, however, results in precipitation of globular proteins (23), whereas centrifugation leads to a considerable loss in protein content (33). If specific steroid binding proteins are present in saliva the preparation of samples before assay may greatly influence the results of steroid hormone determinations, especially when direct assays are used. We were interested whether the method of pretreatment salivary samples (centrifugation or sonification) would influence the concentrations of several steroid hormones, which were assayed with highly specific RIA after extraction and chromatography.

MATERIALS AND METHODS

Subjects

For this study 111 healthy, non-medicated subjects volunteered for the collection of saliva. The group consisted of 20 men, 17 pregnant women and 74 non-pregnant women, aged 15 - 60 year.

Sample collection

Each subject provided 15-20 ml of saliva. Citric acid grains were applied to the tongue to stimulate flow and the saliva was spat into plastic cups. After sampling, the saliva was immediately frozen and kept at -20°C until analysis.

Sample preparation

The saliva samples were thawed at room temperature. After thorough mixing, each sample was divided in two portions. One portion was centrifuged for 10 minutes at 1500 g, which yielded a clear saliva supernatant. The other portion was sonificated (2 times 30 secondes) with a MSE Soni-prep 150 (MSE Scientific Instruments, Manor Royal, Crawbey, Sussex, England) and was referred to as whole saliva.

Materials

Chromatography solvents were of "Baker Analyzed" grade (J.T. Baker Chemical Co., Phillipsburg,NJ). All other chemicals were analytical grade. The pure steroids were purchased from Steraloids (Steraloids INC., Wilton, NH). Tritiated cortisol, cortisone, testosterone, progesterone, androstenedione and estradiol were purchased from New England Nuclear Corp., Boston, MA. Tritiated 17a-hydroxy-progesterone was from Amersham Int. PLC, Amersham, England. The cortisol and cortisone antisera were a gift from Prof.Vecsei, Heidelberg, FDR; testosterone antiserum was a gift from Dr.Pratt, Groningen, The

Netherlands; progesterone and estradiol antiserum were from Organon, Oss, The Netherlands; androstenedione antiserum was purchased from Radio Assay Systems Laboratories Inc., Carson, CA; 17-hydroxy-progesterone antiserum was raised in sheep in the endocrinology division. More details are shown in table I.

Procedures

Salivary steroid concentrations were measured by RIA after extraction and paper chromatography. The methods used have been previously described in detail (3,6,7,34). Aliquots (3 ml) of saliva were incubated with tritiated recovery tracer (± 10,000 dpm each), which was used to monitor procedural losses. After extraction of the samples, the dried extracts were applied to Whatman no.1 paper and chromatographed in the appropriate descending Bush solvent system: Bush B5 toluene/methanol/water 2:1:1 (v/v) for cortisol and cortisone; Bush B3 light petroleum (80-110°) /toluene/methanol/water 333:167:400:100 (v/v) for progesterone, testosterone and estradiol; modified Bush A light petroleum (80-110°)/methanol/water 9:7:4 (v/v) for 17-hydroxyprogesterone and androstenedione. The tritiated hormone areas were located by radioscanning and eluted. The eluates were used for recovery counting and RIA.

The in-house RIA procedures were optimized for the very low concentrations of the steroids in saliva. Dextran-coated charcoal was used for separation of the free and antiserum bound fraction. The analytical variables are summarized in table II.

Statistics

For the statistical analysis of the results we used Pearson regression analysis and Wilcoxon signed rank test.

Table I.

Tracers and antisera: HS = hemisuccinate, CET = carboxy-ethyl-thioether, CME = carboxy-methyl-ether, CMO = carboxy-methyl-oxime, BSA = bovine serum albumin.

Tracer	Specific activity	Immunogen	Antiserum titer
[1,2,6,7-^{3}H]-Cortisol	83.5 Ci/mmol	Cortisol-21-HS-BSA	22,500
[1,2-^{3}H]-Cortisone	29 Ci/mmol	Cortisone-21-HS-BSA	15,000
[1,2,6,7-^{3}H]-Testosterone	102 Ci/mmol	Testosterone-7a-CET-BSA	99,000
[1,2,6,7-^{3}H]-Progesterone	90 Ci/mmol	Progesterone-11-HS-BSA	862,000
[1,2,6,7-^{3}H]-Hydroxyprogesterone	55 Ci/mmol	11-Deoxycortisol-21-HS-BSA	336,000
[1,2,6,7-^{3}H]-Androstenedione	85 Ci/mmol	Androstenedione-19-CME-BSA	7,600
[1,2,6,7-^{3}H]-Oestradiol	104 Ci/mmol	Oestradiol-6-CMO-BSA	88,000

Table 2.

Analytical variables: Bush = chromatography system (see Methods); % Bo = % binding without standard; % NSB = % non-specific binding; Blanco = water blank of the assay (pg/tube); Detection limit = detection limit of the assay (pg/tube) (3xSD of the Bo); Standards = range of standard curve (pg/tube); Tracer = tracer used for recovery and RIA (see tabel 1); Buffer = buffer solution for tracer and antiserum; A = 0.1 M borate buffer, 1 g/L beriglobin, pH 8.0; B = 0.1 M borate buffer, 0.5 g/L bovine serum albumin, pH 8.0.

Steroid	**Cortisol**	**Cortisone**	**Testosterone**	**Progesterone**	**Hydroxy-progesterone**	**Androstene-dione**	**Oestradiol**
Chromatography	Bush B5	Bush B5	Bush B3	Bush B3	Bush A	Bush A	Bush B3
% Bo	51	37	33	41	38	45	42
% NSB	1.3	1.3	3.1	4.3	1.8	6.0	2.2
Blanco	2.1	1.1	0.3	5.0	5.4	3.6	1.2
Detectionlimit	2.0	4.9	1.4	2.2	1.4	2.3	0.9
% Recovery	69	69	43	61	56	44	35
Standards	10-800	10-800	0.5-200	0.5-200	0.5-200	0.5-200	0.5-200
Tracer	4x^{3}H	2x^{3}H	4x^{3}H	4x^{3}H	4x^{3}H	4x^{3}H	4x^{3}H
Buffer	A	A	B	B	A	A	A
Intra-assay % CV	5.9	5.2	7.1	6.7	6.3	5.0	11
Inter-assay % CV	4.9	13	7.7	8.1	6.4	8.6	10

Table 3.

*Concentrations (mean ± SEM) of steroids in whole saliva and supernatant saliva: n = number of subjects, r= correlation (Pearson) between individual steroid levels in the whole saliva and supernatant saliva assay, * = p < 0.0001.*

Steroid		**Whole saliva**			**Supernatant saliva**			
		n	*Mean ± sem*	*range*	*n*	*Mean ± sem*	*Range*	*r*
Progesterone	(nM/L)	64	0.90 ± 0.17	0.03-5.54	60	0.49 ± 0.10	0.02-3.39	0.9735*
Cortisol	(nM/L)	82	8.14 ± 0.88	0.30-35.4	80	8.10 ± 0.93	0.30-43.0	0.9862*
Cortisone	(nM/L)	84	19.2 ± 1.24	1.30-49.2	81	17.6 ± 1.24	1.40-47.7	0.9529*
Androstenedione	(nM/L)	46	0.96 ± 0.13	0.10-3.60	46	1.13 ± 0.17	0.10-4.70	0.9830*
Hydroxyproges-terone	(nM/L)	47	0.17 ± 0.03	0.01-1.20	46	0.16 ± 0.03	0.01-1.25	0.9729*
Testosterone	(nM/L)	54	0.13 ± 0.02	0.014-0.59	50	0.12 ± 0.018	0.01-0.43	0.9827*
Oestradiol	(pM/L)	72	43.4 ± 7.3	2.6-314	65	29.5 ± 4.7	2.0-228	0.9618*

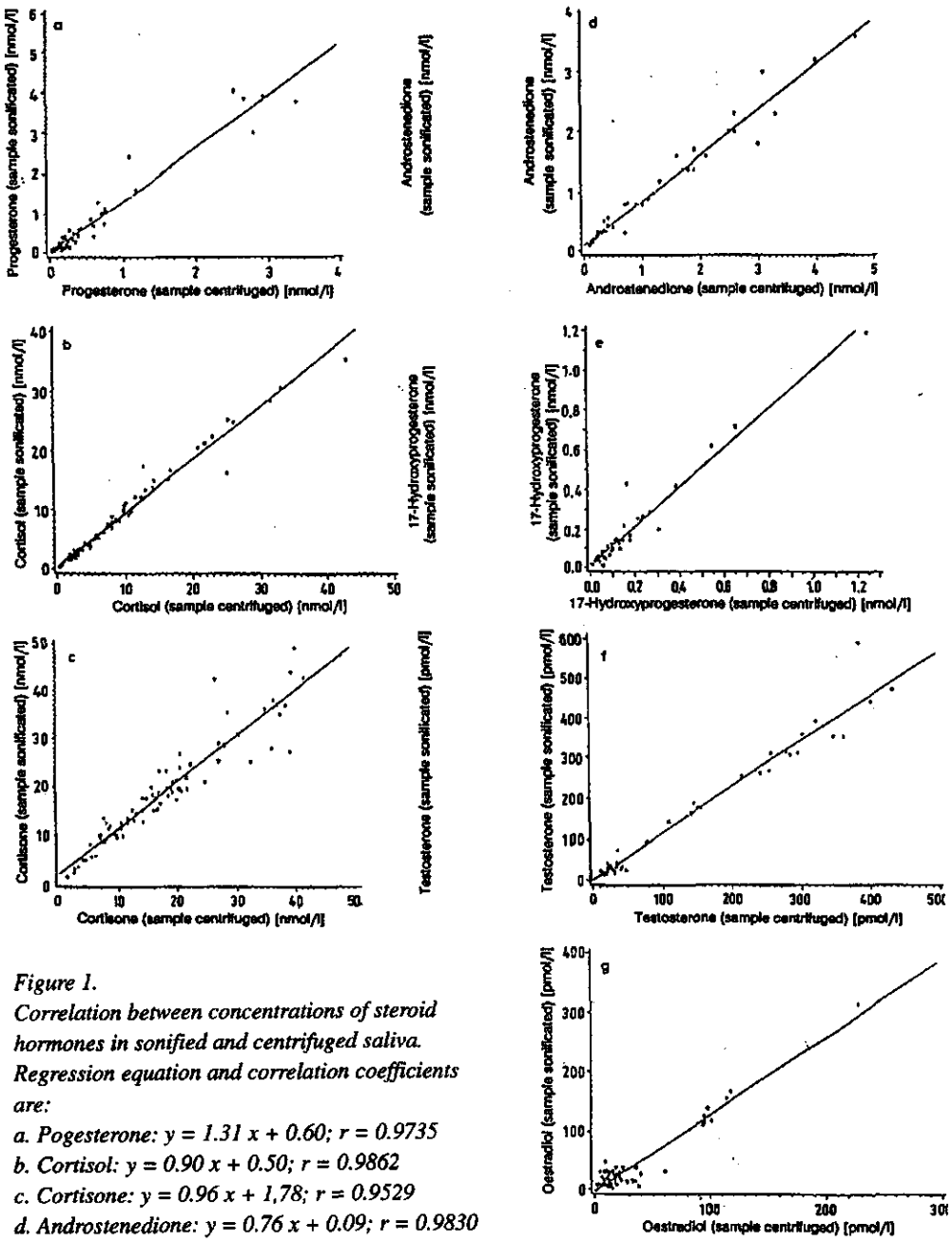

Figure 1.
Correlation between concentrations of steroid hormones in sonified and centrifuged saliva. Regression equation and correlation coefficients are:

a. Pogesterone: y = 1.31 x + 0.60; r = 0.9735
b. Cortisol: y = 0.90 x + 0.50; r = 0.9862
c. Cortisone: y = 0.96 x + 1,78; r = 0.9529
d. Androstenedione: y = 0.76 x + 0.09; r = 0.9830
e. 17-Hydroxyprogesterone: y = 0.99 x + 0.02; r = 0.9729
f. Testosterone: y = 1.14 x + 1.89; r = 0.9827
g. Oestradiol: y = 1.31 x - 2.50; r = 0.9618.

RESULTS

The results of the determinations of the steroids in whole saliva and in supernatant saliva are presented in table 3. Correlations between concentrations in whole saliva and supernatant saliva were all highly significant.

In order to demonstrate differences between the concentrations of the several steroid hormones in whole sailva and supernatant saliva, we calculated for each subject the relative increase after sonification of the samples (supernatant saliva = 100 %). These data are shown in table 4.

Table 4.
Relative difference between whole saliva and supernatant saliva (mean ± SEM): n = number of subjects, P = probability of Wilcoxon signed rank test, ns = not significant.

Steroid	n	Increase(%)	P
Progesterone	59	36 ± 0.06	< 0.0001
Cortisol	80	-1 ± 0.01	ns
Cortisone	81	10 ± 0.02	< 0.0001
Androstenedione	44	-4 ± 0.03	ns
Hydroxyprogesterone	46	17 ± 0.06	< 0.0025
Testosterone	50	22 ± 0.05	< 0.0001
Oestradiol	56	49 ± 0.15	0.02

It appeared that sonification of saliva samples resulted in a statistically significant increase in concentrations of the steroid hormones, with the exception of cortisol and androstenedione. Our study group, however, was quite heterogeneous. The subjects were divided in a group of pregnant women, non-pregnant women and men. In the pregnant group the results for progesterone and estradiol were higher in sonificated than in centrifuged saliva (increase 46 ± 10 %, $p < 0.0001$ and 29 ± 4 %, $p < 0.001$, respectively), whereas for the other steroids there was no significant difference. The values for estradiol in non-pregnant female saliva show differences between sonificated and centrifuged samples as similar to those observed in pregnancy samples; but this interpretation is preliminary, because despite very low sensitivity the levels approximate the detection limit of the assay. In male saliva the most striking difference between whole saliva and supernatant saliva was a decrease (14 ± 4 %, $p < 0.005$) in androstenedione values after sonification of saliva, which was not observed for female non-pregnant saliva.

DISCUSSION

The present study clearly demonstrates the effect of sample preparation on concentrations of steroid hormones measured in saliva. In our study group as a whole, sonification of saliva yielded significantly higher levels of steroids than centrifugation, with the exception of cortisol and androstenedione. For these latter hormones the method of sample preparation had no influence on the results. Considering the groups of pregnant women, non-pregnant women and men separately, the most striking observation was a decrease in androstenedione concentrations in male saliva after sonification. It is possible that sonificating of the saliva samples disperses some unknown substance(s), which interferes in the RIA. It is worth mentioning the results of Baxendale et al.(1982) (4), who demonstrated a sex-dependent difference in salivary testosterone values when comparing chromatographed and direct assays.

In the non-pregnant and male groups the effect of sample preparation was rather similar to that in the study group as a whole. In contrast, in the pregnant group, sonification of saliva resulted in higher values only for progesterone and estradiol. The data for progesterone are comparable to those of Lequin et al.(1986) (17) and indicate a loss of steroid hormone when saliva is centrifuged. It is possible that the pelleted debris of saliva contains most of the binding proteins, which are precipitated by the process of freezing and thawing of saliva (23), whereas centrifugation leads to a further loss of such proteins (33).

Our findings indicate that caution is warranted in the interpretation of data from salivary steroid hormone determinations. If indeed specific steroid binding proteins are present in saliva (31) the question of whether the salivary hormone concentrations reflect the plasma free levels has to be re-opened. The question arises as to which of of the analysed materials, whole saliva or supernatant saliva, gives a real indication of the plasma free level. Assuming that the decrease in steroid concentrations in supernantant saliva is caused by the removal of specific binding proteins, we tend to believe that the latter is the better candidate, despite lower absolute values for the steroid hormones.

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CHAPTER 5

Differences between concentrations of salivary cortisol and cortisone and of free cortisol and cortisone in plasma during pregnancy and postpartum

Differences between concentrations of salivary cortisol and cortisone and of free cortisol and cortisone in plasma during pregnancy and post-partum

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ABSTRACT

We measured cortisol and cortisone - both total and free - in plasma and in saliva during the course of pregnancy and postpartum. Antepartum and postpartum concentrations and morning and afternoon concentrations of both steroids were compared. The mean concentrations of cortisol and cortisone increased towards term and were significantly greater at the end of pregnancy than postpartum, except for free cortisol in plasma in the afternoon. The daily rhythm of both steroids was maintained throughout pregnancy and postpartum. The correlations between salivary and plasma free concentrations of cortisol and cortisone as well as of the sum of cortisol + cortisone were highly significant. The mean concentrations of cortisone in saliva accurately reflected both total and free concentrations in plasma. For cortisol, however, the change of the concentrations in saliva, was somewhat different from that in plasma. More-over, the mass ratio of plasma free cortisol to salivary cortisol was about 2, whereas for cortisone the ratio was only about 0.5, probably owing to the conversion of cortisol to cortisone by 11ß-hydroxysteroid dehydrogenase in the salivary gland. Furthermore, the passage of cortisol and cortisone from plasma to saliva should not be regarded as simple diffusion, because in the first half of pregnancy the sum of the salivary concentrations of cortisol and cortisone was significantly greater then the sum of their free concentrations in plasma.

INTRODUCTION

Measurement of cortisol in saliva is increasingly considered a viable alternative to measurement in plasma. Numerous publications have proven the applicability of salivary cortisol determinations in clinical tests for adrenal activity and in psychological studies as an index for stress or endogenous depression (for reviews, see 1,2). The high correlation between salivary and plasma free cortisol is independent of changes in corticosteroid binding globulin (CBG) concentration (2-4), whereas the relationship between salivary and plasma total cortisol displays nonlinearity. At high concentrations of cortisol, when CBG binding sites become saturated, the concentrations of free cortisol in plasma, and hence of salivary cortisol, increase proportionally more rapidly than at low concentrations. When CBG and cortisol levels are increased concomitantly, e.g., in pregnancy, concentrations of salivary cortisol appear to be normal (3,5-7), although above-normal concentrations have also been reported (8-10). In a longitudinal study, Stahl & Dörner (10) demonstrated that mean salivary concentrations of cortisol gradually increased, exceeding the normal range in

the second part of pregnancy. Vining et al.(3), measuring the daily rhythm of salivary cortisol, showed that in the third trimester of pregnancy salivary cortisol concentrations in morning samples still overlap normal values, whereas in the afternoon samples the pregnancy values are greater.

Since the earliest work of Shannon et al. (11,12) on 17-hydroxycorticosteroids in saliva, it has been known that the concentration of cortisol in saliva comprises only about 50-60% of the concentration of free cortisol in plasma, because the metabolizing enzyme 11ß-hydroxysteroid dehydrogenase (11ßHSD; EC 1.1.1.146) in the salivary gland (9), converts cortisol into cortisone. Despite this substantial conversion, the correlation between salivary and plasma free cortisol is maintained in physiological conditions. CBG is also the high-affinity binding protein for cortisone, but with an association constant about 10 times lower than for cortisol; consequently, there is a much higher proportion of free cortisone than of free cortisol in plasma (13-15). This high free fraction of cortisone in plasma and the substantial conversion of cortisol into cortisone in the salivary gland may explain why the concentrations of cortisone in saliva exceed those of cortisol (4,8,16,17), and seem more closely related to free cortisol in plasma than to free cortisone in plasma (4,15,16).

During pregnancy the placenta is an additional source of plasma cortisone (18). Very little is known about the influence of this extra synthesis of cortisone on the ratio of cortisol to cortisone in plasma or saliva. According to Beitins et al.(19), total cortisone is increased in plasma during pregnancy, yet the ratio of cortisol to cortisone is greater than in non-pregnant women owing to an relatively greater metabolic clearance of cortisone in pregnancy. Others (20,21), however, did not report an increase of total cortisol over cortisone in plasma. Almost no data are available regarding the ratio of free cortisol to cortisone in plasma. From the results of Dunn et al.(13) one can calculate that the concentration of free cortisone in plasma is higher in pregnant women then in normally cycling women, in contrast to the concentration of free cortisol in plasma. With regard to salivary concentrations of cortisol and cortisone, Greaves & West (8) showed that in the third trimester of pregnancy salivary cortisol is twice that of values during nonpregnancy, whereas salivary cortisone is increased threefold. The difference in increases of salivary cortisol and cortisone at the end of pregnancy may result from a considerable increase in free cortisone in plasma and(or) a higher metabolic conversion rate of cortisol into cortisone by 11ßHSD in the salivary gland at this time.

In this study we dealt primarily with the course during pregnancy of total and free concentrations in plasma and of salivary cortisol and cortisone and their inter-relationhips. We were further interested in the interaction between cortisol and cortisone in view of the metabolic interconversion during passage of both steroids from blood to saliva.

MATERIALS AND METHODS

Subjects and Sampling

Thirty-six healthy pregnant women, attending the Gynecologic Clinic of the St. Jozef Hospital in Doetinchem, volunteered in this study. The study protocol was approved by the hospital ethical committee.

Matched blood and saliva samples were taken four times during pregnancy - during weeks 14-19 (period 1), weeks 20-26 (period 2), weeks 27-34 (period 3), and weeks 35-40 (period 4) - and once at six weeks postpartum (period 5). We collected blood and saliva specimens from 19 women at 0900-1000 hours, and from 17 women at 1500-1700 hours. Blood was drawn into heparinized tubes and centrifuged (5 min at 1500 X g), and the plasma was stored at -20 °C. Citric acid-stimulated saliva was collected in plastic cups and stored at -20 °C. Before use , it was thawed and centrifuged (10 min 1500 X g), and the supernatant liquid was used for analysis.

Methods

Concentrations of cortisol and cortisone in plasma (total and free) and in saliva were measured by previously described RIA procedures (4). In short, 0.2 mL of plasma or 0.7 mL of saliva was extracted with dichloromethane, the steroids were purified via paper chromatography in a descending Bush B5 solvent system (toluene/methanol/water, 2/1/1 by vol) (22), and the paper eluates were analyzed by RIA. Procedural losses were controlled for by including ^{3}H-tracers.

Steroid specificity was optimized by using the extraction and paper chromatography methods. Sensitivity of the RIA was 4 pg/tube, resulting in detection limits of 2300, 53 and 84 ng/L for plasma total, plasma free, and salivary cortisol, respectively. The respective detection limits for cortisone were 400, 160 and 100 ng/L. The inter- and intra-assay coefficients of variation were <11% and <6%, respectively.

The concentrations of free cortisol and cortisone in plasma were determined by equilibrium dialysis, followed by the same RIA of cortisol and cortisone in the dialysate (0.3 mL) (4). Inter- and intra-assay coefficients of variation were <7% and <5%, respectively.

All samples from each study subject were assayed in the same run to eliminate effects of interindividual variations.

Statistics

For statistical analysis we used Pearson regression analysis, Student's t-test, Bonferoni's t-test and multiple stepwise regression analysis.

Results

Figure 1 shows the temporal changes in concentrations of free and total cortisol and cortisone in plasma and in saliva, expressed as mean ± SEM for each sampling period.

CONCENTRATIONS IN PLASMA.

Total cortisol and cortisone.

At almost all times the mean concentrations of total cortisol and cortisone in plasma were significantly higher during pregnancy than after parturition, both in the morning and in the afternoon. In the latter stages of pregnancy the mean values were twice (cortisol) or thrice (cortisone) the nonpregnant values. At all times the diurnal rhythm was maintained, as reflected in the significantly higher values for both cortisol and cortisone in the morning than in the afternoon.

Free cortisol and cortisone.

By the end of pregnancy, the mean concentration of free cortisol in plasma had increased to about 1.5 times the nonpregnant or postpartum value, both in the morning and in the afternoon (Figure 1). However, in the morning samples, free cortisol was significantly higher than postpartum values only in the second half of pregnancy, whereas in the afternoon samples the difference between pregnant and nonpregnant values was not statistically significant.

For free cortisone in plasma increases were comparable with those for total cortisone concentrations (about 2.5 times the non-pregnant values) in the morning as well as in the afternoon.

The difference between morning and afternoon concentrations of free cortisol and cortisone in plasma was even more pronounced ($P < 0.0001$) than for the total cortisol and cortisone concentrations, especially in the second half of pregnancy.

SALIVARY CORTISOL AND CORTISONE.

At the end of pregnancy the mean concentration of salivary cortisol had increased to about 1.5 times the postpartum values in the morning and to about 2.5 times the postpartum values in the afternoon.

Mean salivary cortisone concentrations also were significantly increased at the end of pregnancy, being double the nonpregnant values in the morning and triple in the afternoon. For both salivary cortisol and cortisone the daily rhythm was evident in all periods.

PLASMA FREE VS SALIVARY CORTISOL AND CORTISON

Ratio plasma free over salivary cortisol.

The ratios of plasma free over salivary cortisol for each period in morning and afternoon samples are shown in Table 1. The ratio of plasma free over salivary cortisol in the morning samples increased in the course of pregnancy. In the first period of pregnancy in the morning this ratio was significantly lower than postpartum, but afterwards it increased to values comparable to postpartum values. In the afternoon samples the ratio of plasma free over salivary cortisol during pregnancy was significantly lower than postpartum. In the non-pregnant period the ratio of plasma free over salivary cortisol in the afternoon was higher than in the morning.

The ratio plasma free over salivary cortisone.

The ratio of plasma free over salivary cortisone, as shown in Table 1, increased towards term in the morning samples, and at the end of pregnancy it was significantly higher then the postpartum ratio. The afternoon samples showed no statistically significant difference between mean ante- and postpartum values. In the non-pregnant period the ratio of free plasma to salivary cortisone in the afternoon was higher than in the morning samples.

Table 1.
Ratios of concentrations of cortisol and cortisone free in plasma to those in saliva

	Morning samples		**Afternoon samples**	
Period[a]	*Ratio*[b]	*n*	*Ratio*[b]	*n*
Cortisol				
1	1.72 ± 0.09	20	1.88 ± 0.11[c]	17
2	1.78 ± 0.08	18	2.10 ± 0.15[c]	17
3	2.11 ± 0.11	19	2.38 ± 0.29[c]	18
4	2.46 ± 0.34	17	2.28 ± 0.11[c]	15
5	2.32 ± 0.20	17	3.53 ± 0.38	15
Cortisone				
1	0.46 ± 0.02	20	0.50 ± 0.03	17
2	0.44 ± 0.02	18	0.47 ± 0.04	18
3	0.51 ± 0.03	19	0.60 ± 0.06	18
4	0.59 ± 0.04*	17	0.61 ± 0.03	15
5	0.44 ± 0.03	17	0.63 ± 0.07	16

[a] 1, 14-19 weeks of gestation; 2, 20-24 weeks; 3, 25-34 weeks; 4, 35-40 weeks; 5, six weeks postpartum. [b] Mean ±SEM. [c] Significantly different from period 5 (P<0.05, Bonferroni t-test).

Table 2.
Cortisol + cortisone concentrations and ratios (Means ± SEM)

Cortisol + cortisone

Period	*n*	*Plasma(free) µg/L*	*Saliva µg/L*	*Plasma(free): saliva ratio*
Morning samples				
1	20	11.36 ± 0.55	14.35 ± 0.78[a]	0.81 ± 0.03[de]
2	19	14.97 ± 0.91[d]	17.29 ± 1.26[b,d]	0.86 ± 0.03[e]
3	19	17.79 ± 0.64[d]	19.02 ± 0.99[d]	0.96 ± 0.04
4	15	21.74 ± 1.05[d]	22.90 ± 1.09[d]	1.01 ± 0.07
5	17	11.83 ± 1.09	12.62 ± 0.93	0.98 ± 0.06
Afternoon samples				
1	17	5.40 ± 0.38	6.27 ± 0.27[c]	0.85 ± 0.04[d,e]
2	17	7.19 ± 0.73[d]	8.46 ± 0.65[d]	0.87 ± 0.06
3	18	8.69 ± 0.69[d]	8.90 ± 0.70[d]	1.04 ± 0.13
4	15	11.09 ± 0.43[d]	11.88 ± 0.50[d]	0.95 ± 0.04
5	16	6.24 ± 0.79	4.78 ± 0.41	1.27 ± 0.14

Periods as in Table 1. [a,b,c] Significantly different from free plasma concentrations (paired t-test: [a] P<0.0001; [b] P<0.001; [c] P<0.01; d significantly different from period 5 (postpartum): P<0.05 (Bonferroni t-test); [e] ratio significantly different from 1.0 (t-test).

Table 3.
Correlation between total plasma, free plasma, and salivary cortisol and cortisone

Comparison *x vs y*	**Regression equation** $y = ax + b$	r^a	*n*
Morning samples			
TF vs FF	y = 0.020 x + 3.21	0.7552	93
TF vs SF	y = 0.011 x + 1.77	0.5513	93
FF vs SF	y = 0.432 x + 1.00	0.5949	93
TE vs FE	y = 0.168 x + 0.095	0.9059	92
TF vs SE	y = 0.235 x + 4.14	0.7312	91
FE vs SE	y = 1.295 x + 4.64	0.7450	91
SE vs FF	y = 0.88 x + 3.76	0.6958	91
CS vs CF	y = 0.97 x + 2.31	0.8017	91
TF vs FF	y = 0.018 x + 0.69	0.6947	85
TF vs SF	y = 0.009 x + 0.16	0.7319	83
FF vs SF	y = 0.324 x + 0.55	0.6965	83
TE vs FE	y = 0.108 x + 0.61	0.8474	86
TE vs SE	y = 0.150 x + 2.23	0.7829	85
FE vs SE	y = 1.163 x + 2.16	0.7714	85
SE vs FF	y = 0.67 x + 3.08	0.5705	85
CS vs CF	y = 0.73 x + 2.35	0.7548	84

Intercept in micrograms per liter. TF, total plasma cortisol; FF, free plasma cortisol; SF, salivary cortisol;
TE, total plasma cortisone; FE, free plasma cortisone; SE, salivary cortisone; CF, free plasma cortisol + cortisone; CS, salivary cortisol + cortisone; n, number of samples; r = Pearson correlation coefficient; [a] all correlations significant at $P < 0.0001$.

SUM OF CORTISOL AND CORTISONE IN PLASMA AND SALIVA.

Because of the close interrelationship between cortisol and cortisone, we were interested to know whether the sum of the concentrations of free cortisol + cortisone in plasma were comparable to the sum of the salivary cortisol + cortisone concentrations. These results are shown in Table 2.
It appeared that the mean concentrations of free cortisol + cortisone in plasma as well as in saliva increased during pregnancy to about twice the postpartum value. In the second half of pregnancy the concentration of free cortisol + cortisone in plasma was virtually completely matched in saliva. Surprisingly, in the first half of pregnancy, values for cortisol + cortisone in the saliva were greater than could be accounted for by the free concentrations in plasma. The ratio of the concentrations of free cortisol + cortisone to salivary cortisol + cortisone at that time was significantly < 1.

RELATION BETWEEN FREE CORTISOL AND CORTISONE IN PLASMA AND IN SALIVA

Correlations between plasma free and salivary concentrations.

The relation between the concentrations of free cortisol and cortisone in plasma and salivary cortisol and cortisone was determined with Pearson regression analysis and the results are shown in Table 3.
There was a statistically significant correlation between free plasma and salivary cortisol in the morning samples as well as in the afternoon samples. An even better correlation was found between free plasma and salivary cortisone for both the morning adn the afternoon samples.
The correlation between salivary cortisone and free cortisol in plasma was somewhat less tight at both sampling times. As was to be expected, the closest relation existed between the concentrations of free cortisol + cortisone in plasma and salivary cortisol + cortisone, both in the morning and in the afternoon.

Stepwise regression analysis.

To determine the overall dependency of salivary cortisol on the corresponding plasma total and free parameters, we applied multiple stepwise regression analysis (Table 4). This procedure revealed that salivary cortisol in morning samples correlated with salivary cortisone, free cortisol in plasma, and free cortisone in plasma in an ascending order of partial correlation coefficient with P-values < 0.05. In the afternoon samples, salivary cortisol correlated with salivary cortisone, free cortisol in plasma, free cortisol + cortisone in plasma, and total cortisol in plasma. Applying the same procedure for salivary cortisone, it appeared that, in morning samples, salivary cortisone correlated with salivary cortisol and free cortisone in plasma. In the afternoon samples the order of the parameters was total cortisone in plasma, salivary cortisol, and free cortisone in plasma.
It can be concluded that the concentration of cortisol in saliva depends predominantly on salivary cortisone and, surprisingly, less on plasma free cortisol. The concentration of corti-

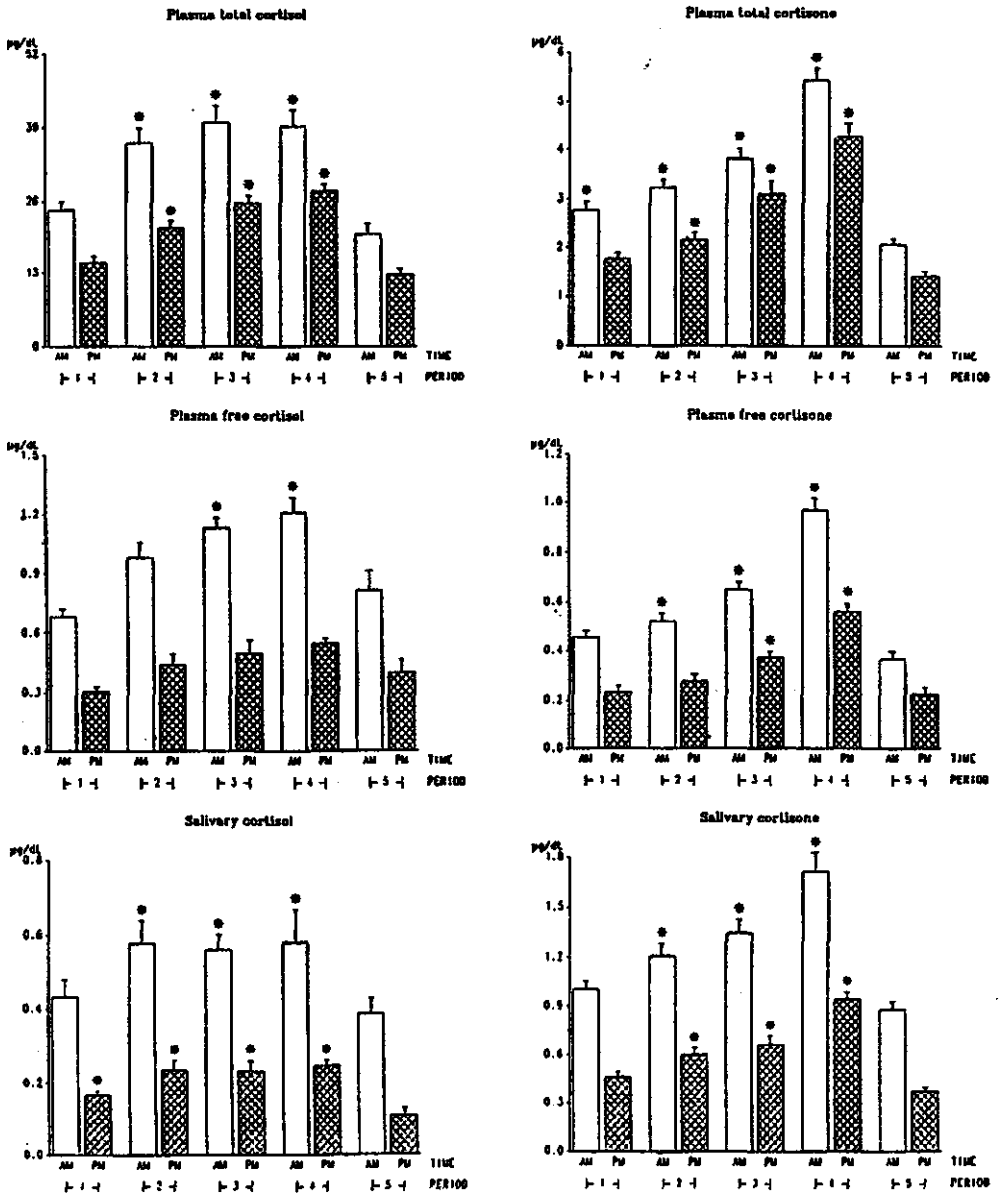

Figure 1.
Concentrations of plasma total, plasma free and salivary cortisol (left panel) and cortisone (right panel) during the course of pregnancy.
*Mean (± SEM) concentrations during pregnancy and at six weeks postpartum; * = significantly different from postpartum ($P < 0.05$, Bonferroni t-test). Periods as defined in Materials and Methods.*

sone in saliva depends strongly on salivary cortisol in the morning, but more on total cortisone in plasma in the afternoon.

Table 4.
Multiple stepwise regression analysis.

Morning samples			**Afternoon samples**		
Variables	*Rp*	*P*	*Variables*	*Rp*	*P*
SF vs SE	0.734	0.0001	SF vs SE	0.731	0.0001
FF	0.243	0.0005	FF	0.328	0.001
FE	0.199	0.005	CF	0.219	0.007
			TF	0.149	0.02
SE vs SF	0.734	0.0001	SE vs TE	0.781	0.0001
FE	0.458	0.0001	SF	0.409	0.0001
			FE	0.123	0.02

Abbreviations as in Table 3; Rp = partial correlation coefficient.

DISCUSSION

During pregnancy, concentrations of total and free cortisol and cortisone in plasma increase to two and three times postpartum values, respectively. The daily rhythm, evident for cortisol as well as for cortisone, is maintained until term. Remarkably, the difference between morning and afternoon concentrations of total cortisol in plasma becomes less at the end of pregnancy, in contrast to the mean concentrations of free cortisol in plasma and salivary cortisol.

Comparing the mean concentrations of free cortisol and cortisone in plasma at the end of pregnancy (period 4) with those postpartum (period 5), it is evident that free cortisone in plasma is significantly greater in period 4 in the morning as well as in the afternoon, whereas for cortisol only in the morning samples a significant difference was found. The morning values are in line with the view of many authors (23-34) of a hypercortisolism at the end of pregnancy at the level of the free concentration in plasma. In the afternoon, however, concentrations of free cortisol in plasma were not significantly different from the postpregnant values. This contrasts with data of other investigators (35-39). The results of these authors can be critizised on the base of aspecific determinations of total cortisol in plasma or biased measurement of the free fraction of of cortisol in plasma, leading to erroneously high free concentrations in plasma.

In saliva the picture is different for cortisol and cortisone. Cortisone in saliva is a true reflection of the plasma free concentration, but cortisol is not, because the mean concentra-

tions of cortisol in saliva show a plateau in the second part of pregnancy, whereas the concentrations of free cortisol in plasma keep increasing. Nevertheless, the mean concentrations of cortisol in saliva were significantly greater in pregnancy than postpartum values, confirming the results of other investigators (6,8-10).

The passage of unconjugated steroids has - until recently - always been regarded as a simple diffusion process through the salivary gland. Chu & Ekins (40), however, reported the presence of CBG in saliva and concluded that salivary steroid concentrations are not identical to their corresponding free concentrations in plasma. With regard to the concentration of cortisol in saliva the relation to free cortisol in plasma is complicated by the conversion of cortisol into cortisone in the salivary gland. The presence of the enzyme 11ß-HSD in the salivary gland was first noted by Katz and Shannon (9), whereas no activity could be demonstrated in liquid saliva (8,9,41), confirmed by our onw experiments (personal observations).

To determine what proportion of free cortisol in plasma appears in saliva, we calculated the ratio of the concentrations of free cortisol in plasma to salivary cortisol in each period (Table 1). For nonpregnant women this ratio was 2.32 ± 0.20 in the morning and 3.53 ± 0.38 in the afternoon, indicating that in the morning relatively more free cortisol in plasma reaches the saliva than in the afternoon, which might be a result of less conversion of cortisol into cortisone. In the first period of pregnancy this ratio was decreased to 1.72 ± 0.09 and 1.88 ± 0.11, respectively, being statistically different from postpartum ($P < 0.05$, Bonferroni t-test). From these data we conclude that, at least in the first half of pregnancy, cortisol in saliva constitutes a greater part of the corresponding free concentration in plasma than that postpartum. During the latter stages of pregnancy this ratio increased to postpartum values in the morning, whereas in the afternoon the ratio remained below postpartum values.

The picture is quite different for cortisone. The ratio of the concentrations of free cortisone in plasma to salivary cortisone in morning samples increased during pregnancy, the ratio being significantly higher than the postpartum ratio in period 4. This implies that at the end of pregnancy there is relatively less cortisone in saliva than might be expected from the free cortisone concentration in plasma. In the afternoon there was no statistically significant difference in the ratio free plasma to salivary cortisone between all periods.

In our study we also calculated the combined concentrations of cortisol + cortisone free in plasma and in saliva. As shown in Table 2, in the first half of pregnancy the mean concentrations of cortisol + cortisone in saliva were significantly higher than free cortisol + cortisone in plasma (ratio < 1.0), indicating that more cortisol + cortisone reaches the saliva than might be expected from their combined free concentrations in plasma. Relating these data to the ratios of plasma free over salivary cortisol or cortisone, as discussed above, our conclusion is that in the first half of pregnancy the increased concentration of cortisol + cortisone in saliva compared with their free concentrations in plasma is caused predominantly by cortisol.

Note the difference between the concentrations of free cortisol + cortisone in plasma and salivary cortisol + cortisone in the postpartum afternoon samples. Remarkably, less cortisol + cortisone was measured in saliva then could be expected from their corresponding free concentrations in plasma. However, this difference did not reach statistical significance ($P < 0.10$, Student's t-test) and conclusive data await further research.

Summarizing our results, we conclude that pregnancy leads to a hypercortisolism and a hypercortisonism at the level of the free concentrations in plasma, especially in the second half of gestation, which is reflected in saliva. It should be mentioned, however, that when one is measuring corticosteroids in plasma or in saliva it is very important to take into account the daily rhythm of these steroids. When free cortisol is measured in plasma only in the afternoon, a hypercortisolism is no longer demonstrable.

The correlation between of concentrations of cortisol and cortisone free in plasma and in saliva was highly significant. The same holds true for the correlation between free plasma and salivary concentrations of cortisol + cortisone. An also highly significant, but somewhat less tight, correlation exists between free cortisol in plasma and salivary cortisone. Despite an excellent corelation between free cortisol in plasma and in saliva, however, the picture of the courses of the mean concentrations during pregnancy and postpartum shows some discrepancies.

The passage of both steroid from plasma to saliva is not a simple diffusion. Apart from the conversion of cortisol into cortisone by 11ß-HSD in the salivary gland, which is clearly demonstrated in our study, it appears that in the first half of pregnancy the sum of cortisol + cortisone recovered in saliva is significantly greater than could be expected from their free concentrations in plasma. This observation could be explained be a concomitant secretion of CBG and/or albumin in saliva (40). More research is needed to elucidate the mechanisms involved in the passage of cortisol and cortisone from plasma to saliva and it may be interesting to implicate the influence of corticosteroids on salivary blood flow (42) and the possibility of product inhibition at the level of 11ß-HSD metabolism (43).

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CHAPTER 6

The effect of oral contraceptive use and pregnancy on the daily rhythm of cortisol and cortisone

The effect of oral contraceptive use and pregnancy on the daily rhythm of cortisol and cortisone

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SUMMARY

The effect of oral contraceptives and of pregnancy on the daily rhythm of cortisol, and its metabolite cortisone in plasma and saliva has been investigated. In both conditions the total plasma cortisol levels were raised to the same extent, the mean values in saliva in the oral contraceptive users being intermediate between those in pregnancy and in the controls, particularly in the morning. Salivary cortisone levels were more related to salivary cortisol than to total plasma cortisone which exhibited a rather flat daily rhythm. There was a shift in peak values for salivary cortisol and cortisone towards late morning: this may be due to a delay in the daily activation of the hypothalamic-pituitary-adrenal-axis in these subjects.

KEYWORDS:
DIURNAL RHYTHM, SALIVA , CORTISOL, CORTISONE, PREGNANCY, ORAL CONTRACEPTIVES.

INTRODUCTION

The stimulatory influence of estrogens on the synthesis of corticosteroid-binding globulin (CBG) and the related increases in total plasma cortisol during oral contraceptive (OC) usage and in pregnancy have been described [1-6]. The biologically active fraction of cortisol in blood is located in the non-protein-bound moiety of plasma cortisol. While the daily rhythm in free plasma cortisol in pregnancy and OC users is maintained it is questionable whether plasma free cortisol is increased during OC usage or in pregnancy [7-10]. The discrepancies in reported data on free plasma cortisol concentrations are related to the different methods of analysis: these are time consuming and sensitive to experimental conditions such as temperature and dilution [11].
The concentrations of cortisol in saliva are closely related to the free concentrations in plasma and the salivary assay is regarded as an excellent substitute for the free plasma cortisol concentration [12-14]. Salivary cortisol also exhibits a daily rhythm similar to total plasma and free plasma cortisol [15]. Vining et al [16] compared the daily rhythm of cortisol in the saliva of third trimester pregnant women and non-pregnant women. It was concluded that salivary cortisol concentrations at the end of pregnancy were raised above the normal during the day, showing a significant difference in the afternoons.
The enzyme, 11ß-hydroxy-steroid dehydrogenase (11HSD), which is present in the salivary glands converts approximately 50 % of cortisol to cortisone during its passage from plasma

into saliva. Thus, the concentrations of salivary cortisone are probably related more to free plasma cortisol than to free plasma cortisone [17]. The daily rhythm of salivary cortisone, especially during OC usage or in pregnancy has not been reported hitherto. This paucity of data prompted us to investigate matched samples of plasma and saliva obtained from women taking oral contraceptives, pregnant women, and a group of controls.

MATERIALS AND METHODS

Subjects

Twenty six healthy individuals volunteered for the study: 10 were pregnant women, 11 were women using low-estrogen OC's and 12 were non-pregnant women, not using OC, who served as the control group. The women from the pregnancy group who returned postpartum were subsequently classified as controls. The age of the women ranged from 20 to 48 years.

The oral contraceptives used were stediril, microgynon-30, trinordiol and trigynon.

Sampling protocol

All of the volunteers provided citric-acid stimulated saliva samples during a whole day from approx. 05.00 a.m. until midnight. Portions (5 mL) of saliva were collected in plastic cups every 30 min. in the early morning and then at intervals of one or two hours. They were then stored at -20°C until analysed. The saliva samples were thawed, centrifuged (10 min. 1500 X g) and the supernatant used for the assay.

Matched blood from 10 women (2 controls, 3 OC-users and 5 pregnant) was also sampled. An indwelling catheter was used to draw (5 mL) blood into heparinized vacutainer tubes between 07.00 and 15.00 h. The blood was centifuged (5 min. at 1500 x g) and the plasma stored at -20°C prior to analysis. Four pregnant women returned two or three times during their pregnancies and then post partum.

Ten members of the pregnancy group provided salivary samples 2 to 3 times during their pregnancy and from 4 of these we also obtained matched plasma samples.

The individuals in the OC-users group provided saliva, and where possible plasma samples, at the end of the "on-pill" period and at the end of the "off-pill" week.

METHODS

Salivary and total plasma cortisol and cortisone concentrations were measured by RIA, after previous extraction and paper chomatography as described earlier [17]. Plasma free cortisol was determined using equilibrium dialysis (1:1) followed by the same RIA of cortisol in the dialysate [17].

DATA ANALYSIS

Data processing was performed using SAS software (SAS Institute, Carey, NC). The profile of the daily variation in hormone concentrations was characterized by several parameters. Each daily rhythm consisted of about 10 consecutive samples, which provided a peak level and a matched peak time. The area-under-the-curve (AUC) for each daily profile was

calculated using a trapezoidal method (addition of all trapezoidum areas between sampling points) and a standardized AUC (S-AUC), i.e. the AUC per hour. Theoretical peak levels and matched peak times via parabol calculation were also computed since the concentrations of both hormones changed rapidly during the mornings, and because sampling-time intervals were more then 20 min. apart. Group comparison of the data was performed with the ANOVA t-test.

Table I.
Rhythm parameters of salivary and plasma cortisol and cortisone.

	CONTROLS		**OC-GROUP**		**PREGNANCY GROUP**	
	mean ± sem	*n*	*mean ± sem*	*n*	*mean ± sem*	*n*
a) Salivary data via the trapezoidal method:						
SFx	16.4 ± 1.0	12	20.8 ± 1.3	24	23.0 ± 1.4[a]	28
SFt	7.10 ± 0.26	12	7.59 ± 0.17	24	7.92 ± 0.20[a]	28
SFs	5.0 ± 0.5	12	6.4 ± 0.7	24	8.2 ± 0.5[a,b]	28
SEx	32.6 ± 2.4	12	38.5 ± 2.1	24	53.0 ± 3.2[a,b]	28
SEt	7.26 ± 0.24	12	7.90 ± 0.20	24	7.97 ± 0.21[a]	28
SEs	14.1 ± 1.1	12	17.1 ± 1.3	24	26.5 ± 1.6[a,b]	28
b) salivary data via the parabol method:						
SFx-c	17.8 ± 1.5	9	20.8 ± 1.4	21	23.7 ± 1.6[a]	23
SFt-c	7.19 ± 0.24	9	7.72 ± 0.15	21	8.17 ± 0.24[a]	24
SEx-c	33.6 ± 2.9	10	38.2 ± 1.8	24	56.5 ± 3.8[a,b]	22
SEt-c	7.32 ± 0.20	10	7.80 ± 0.15	24	8.32 ± 0.23[a,b]	22
c) plasma data via the trapezoidal method:						
TFx	482 ± 62	4	1089 ± 146[a]	5	1006 ± 103[a]	11
TFt	7.90 ± 0.37	4	8.28 ± 0.70	5	7.89 ± 0.35	11
TFs	261 ± 29	4	679 ± 95[a]	5	703 ± 66[a]	11
TEx	57.1 ± 4.4	4	84.9 ± 8.3	5	116.2 ± 12.8[a]	11
TEt	7.67 ± 0.30	4	10.12 ± 0.75[a]	5	9.65 ± 0.47[a]	11
TEs	34.1 ± 5.8	4	63.2 ± 6.7	5	91.5 ± 9.2[a]	11

n, number of subjects; SFx, salivary concentration of cortisol (nM/l); SFt, matched peak time of salivary cortisol (h); SFs, S-AUC of salivary cortisol (nM/l per h); SEx, salivary concentration of cortisone (nM/l); SEt, matched peak time of salivary cortisone (h); SEs, S-AUC of salivary cortisone (nM/l per h); SFx-c, parabol calculated concentration of salivary cortisol; SFt-c, calculated matched peak time; SEx-c, calculated concentration of salivary cortisone; SEt-c, matched peak time; TFx, concentration of plasma total cortisol (nM/l); TFt = matched peak time of plasma total cortisol; TFs, S-AUC of plasma total cortisol (nM/l per h); TEx, concentration of plasma total cortisone (nM/l); TEt, matched peak time of plasma total cortisone; TEs, S-AUC of plasma total cortisone (nM/l per h). [a] *Significantly different from controls.* [b] *Significantly different from OC-group (t-test). Real clock times in hr:min are derived from the peak time decimal x 0.6.*

RESULTS

Shift in daily rhythm characteristics of salivary and plasma cortisol and cortisone in normal women, women taking oral contraceptives and pregnant women

The concentrations of salivary and total plasma cortisol and cortisone in the three groups are shown in Fig. 1. Elevated levels of salivary cortisol and cortisone were observed in pregnancy and OC-usage ($P < 0.0001$, ANOVA) and the peak time of each hormone was shifted towards later in the morning.

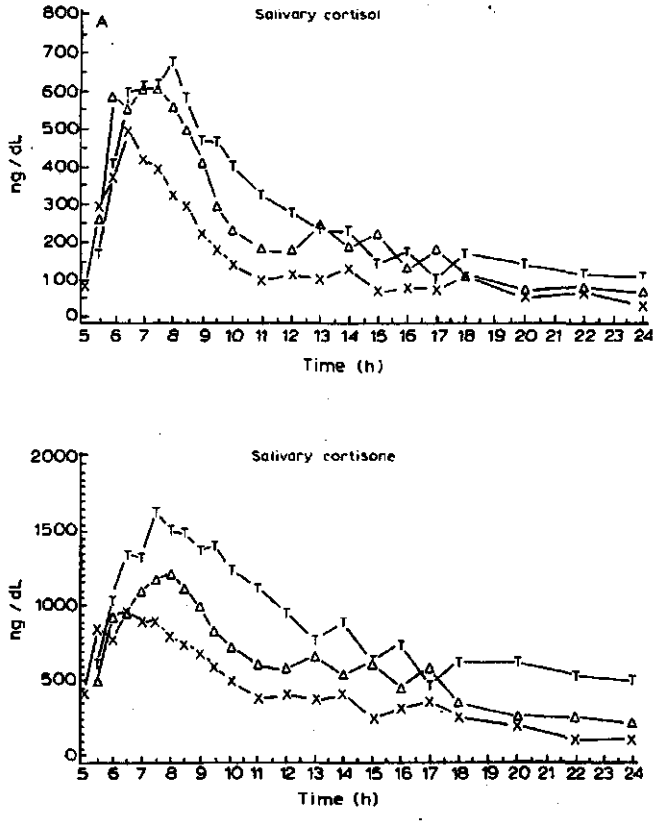

Figure 1.
Daily rhythm of salivary and plasma total cortisol and cortisone. Mean concentrations of (A) salivary (ng/dl) and (B) plasma total (µg/l) cortisol and cortisone; (X—X) control group, (Δ—Δ) OC group, (T—T) pregnant group.

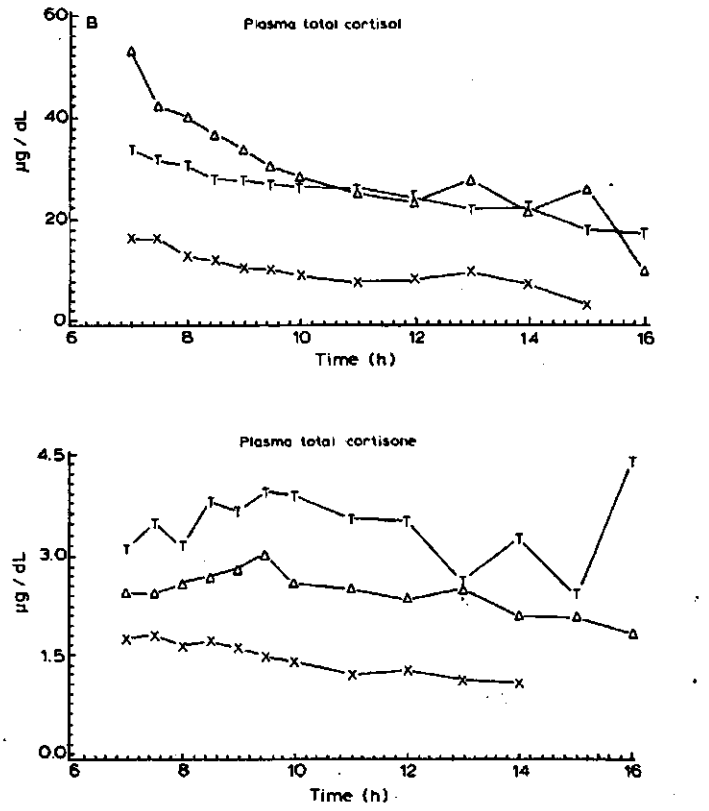

Figure 1, continued

Salivary and plasma cortisol and cortisone concentrations in control women, women using oral contraceptives and pregnant women

The mean (± SEM) peak level, matched peak time, AUC and S-AUC calculated from the daily profiles of salivary and plasma cortisol and cortisone indicated that each of these indices increased significantly during pregnancy (Table Ia-c). The values for these indices in the OC-users group were intermediate between those of the control and the pregnancy groups.

It is evident (Table Ia) that increases in the concentrations of salivary cortisol and cortisone are accompanied by a delay in peak times. Additional supporting evidence was provided by parabol calculation of peak concentrations and matched peak times (Table Ib).

Analysis of the data from plasma demonstrated only slight differences between the OC-users and the pregnancy group, the substantial increase in mean total plasma concentra-

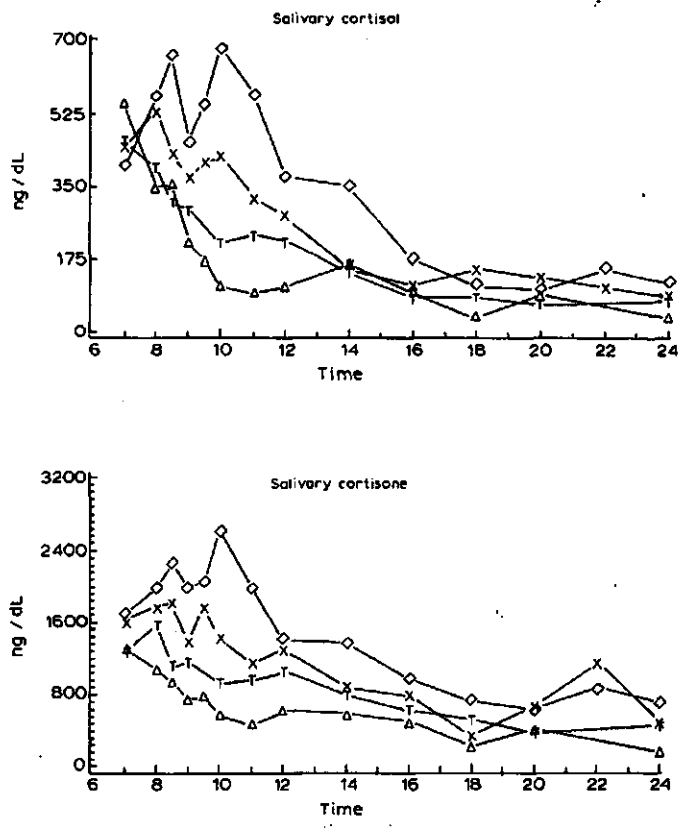

Figure 2.
Daily rhythm in salivary cortisol and cortisone in the course of pregnancy. Concentrations of salivary cortisol and cortisone (ng/dl) in the first (T—T), second (X—X) and third (◇—◇) trimester of pregnancy and 2 months postpartum (Δ—Δ).

tions and S-AUC of cortisol being confirmed (Table Ic). The absence of any marked delay in the plasma cortisol peak times compared to the significant delay of the corresponding cortisone value was noteworthy. It was also shown that an increase in total plasma concentration and S-AUC of cortisone occurred only during pregnancy.

Rhythm parameters of salivary and plasma cortisol and cortisone in pregnancy

The cortisol and cortisone data, divided in values from the first half and the second halves of pregnancy, are shown in Table II. The peak concentrations of salivary and total plasma cortisone, and the matched S-AUC, were significantly higher in the second half of pregnancy. Insignificant statistical differences were detected for the cortisol parameters.

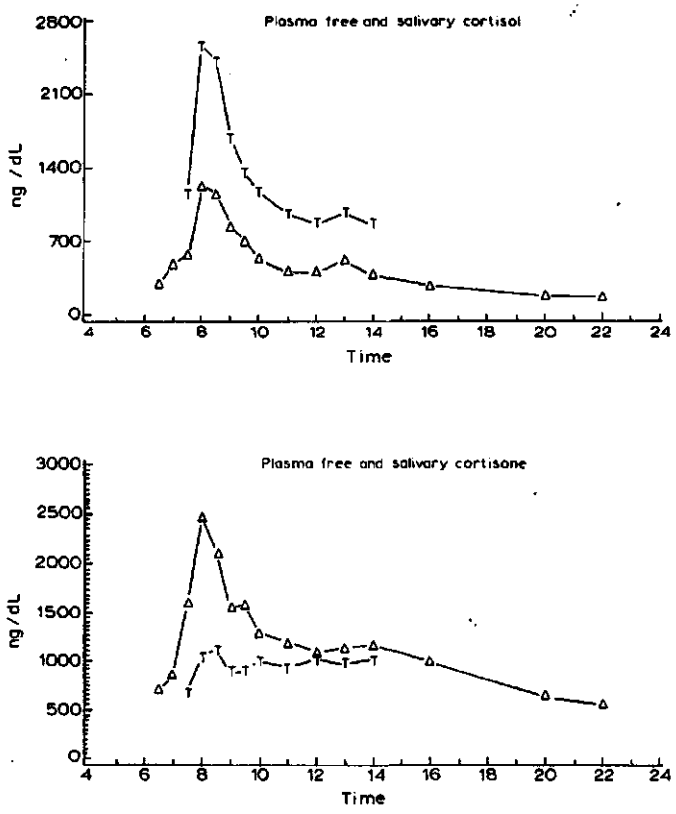

Figure 3.
Relation between salivary and plasma free cortisol and cortisone in one woman.
Concentrations (ng/dl) of salivary (Δ—Δ) and plasma free (T—T) cortisol and cortisone from one subject in the third trimester of pregnancy.

Rhythm parameters of salivary and plasma cortisol and cortisone levels in women using oral contraceptives

Insignificant differences were found between the "on-pill" and the "off-pill" sampling days, although the peak time for salivary cortisol and cortisone showed a tendency to be delayed at the end of the "on-pill" cycle (7.89 ± 0.20 h vs 7.39 ± 0.21 h for salivary cortisol and 7.99 ± 0.18 h vs 7.48 ± 0.25 h for salivary cortisone).

Salivary versus plasma free cortisol and cortisone in pregnant women

Salivary cortisol is thought to be derived preponderantly from plasma free cortisol. The daily rhythm in salivary cortisol and in plasma free cortisol was compared in a few individuals. The curves almost parallelled each other, although the concentrations of salivary cortisol were approximately half the free plasma cortisol.
In contrast, there was a poor relationship between salivary and free plasma cortisone, at least at the end of pregnancy, when cortisone in plasma was high. The daily rhythm of salivary cortisone was then comparable to that of salivary and free plasma cortisol, whereas free plasma cortisone, after an initial morning peak did not decrease until the end of the sampling time (14.00 h).

Table II.
Rhythm parameters of salivary and plasma cortisol and cortisone in pregnancy.

Pregnancy	**First half**		**Second half**	
	Mean ± SEM	*n*	*Mean ± SEM*	*n*
a) Salivary data via the trapezoidal method:				
SFx	22.5 ± 2.5	11	23.3 ± 1.7	16
SFt	7.99 ± 0.35	12	7.86 ± 0.25	16
SFs	7.4 ± 1.1	12	8.7 ± 0.4	16
SEx	45.1 ± 4.8	12	58.7 ± 3.8[a]	16
SEt	7.93 ± 0.32	12	8.01 ± 0.28	16
SEs	20.8 ± 1.9	12	30.8 ± 1.9[a]	16
b) salivary data via the parabol method:				
SFx-c	23.3 ± 3.4	7	23.9 ± 1.8	16
SFt-c	8.62 ± 0.44	8	7.94 ± 0.27	16
SEx-c	47.0 ± 6.7	8	61.9 ± 4.1[a]	14
SEt-c	8.47 ± 0.40	8	8.23 ± 0.29	14
c) plasma data via the trapezoidal method:				
TFx	874 ± 182	3	1056 ± 126	8
TFt	7.56 ± 0.39	3	8.02 ± 0.46	8
TFs	591 ± 118	3	745 ± 78	8
TEx	76.3 ± 5.8	3	131.2 ± 13.9[a]	8
TEt	8.75 ± 0.89	3	9.99 ± 0.53	8
TEs	60.2 ± 4.4	3	103.5 ± 9.4[a]	8

For parameter explanation see Table I; n, number of observations. [a] Significantly different from values in the first half of pregnancy.

DISCUSSION

The present study demonstrates clearly the effects of OC-usage or pregnancy on the daily rhythm of salivary cortisol and cortisone leading to an increase in peak levels and a delay in peak time. In particular, a shift in peak time from ca. 07.00 h. under non-pregnant conditions to ca. 08.00 h. during pregnancy was found to occur. This observed time shift was supported by comparing earlier data of total plasma cortisol during pregnancy and normal values. Analysis of these data indicated a similar shift in the time of appearance of the maximal total plasma concentration of cortisol towards later in the morning. This observation had not been discussed earlier [18-20].

There were no significant statistical differences in the mean plasma concentrations of cortisol and cortisone between the OC-users and the pregnancy group. The salivary rhythm parameters in the OC-users group were however intermediate between the other two groups in the morning, but no deviation from the normal group was observed in the evenings.

In the current study samples were obtained more than once from the pregnant women during the gestation and postpartum period. A steady shift in the peak time to later in the morning during gestation was observed when the individual daily rhythms of cortisol and cortisone were compared (Fig. 2). They returned to early morning in the postpartum period. The cortisol and cortisone parameters (Table II) obtained during the first half of pregnancy were then compared with those of the second half and with the control. These showed that an increase in peak time and concentrations occurred mainly during the first half. This observation supported earlier results [21,22] which suggested a sharp increase in plasma total cortisol and CBG during the first half of pregnancy, followed by subsequent levelling. The largest deviation from normal cortisone values were noted during the second half.

The daily rhythm of cortisol and cortisone at the end of the "on-pill" and "off-pill" periods in the OC-users group were then compared. No significant differences in the measured parameters were observed on these days, although the peak times for salivary and total plasma cortisol and cortisone increased slightly at the end of the "on-pill" period. The individual curves of salivary cortisol and cortisone showed a peak-time shift (ca. 30 minutes) at the end of the "on-pill" period, although there were no significant differences in the concentrations of either hormone.

The results of earlier work [23,24] could help explain the shifts in peak time of the daily rhythm of cortisol and cortisone during OC usage and pregnancy observed in the current study. These earlier studies reported that the increased synthesis of CBG, and concomitant increase in total plasma cortisol during OC usage and pregnancy was estrogen induced. This resulted in a lowered metabolic clearance rate and a higher t1/2 for the metabolism of cortisol. CBG-bound cortisol appeared to have some buffer function in the plasma. Stimulation of HPAA feedback is initiated when the concentration of free plasma cortisol decreases below a minimum value, which is reached some hours prior to morning awakening under normal conditions. There is a delay in reaching this minimum if the buffer capacity of CBG-cortisol is large. A corresponding delay in the activition of HPAA then ensues. A delay in the stimulation of cortisol synthesis by the adrenal glands during pregnancy has also been reported [25]. The daily rhythms of cortisone, a metabolite of cortisol, will likewise parallel this shift.

An elevated level of free plasma cortisol in the two study groups was observed in the mornings when the daily concentration rhythms were compared with the control ($P < 0.05$, ANOVA, n=5; data not included). These returned to the mean normal values during the afternoon. A normal level of free plasma cortisone was observed for OC users, whereas an increased level during pregnancy was maintained throughout the day. The measured data also indicated that free plasma cortisol levels ($P > 0.05$) during pregnancy were not above normal in the afternoons. The levels in saliva were however raised ($P < 0.001$) both in the afternoons and evenings. The increased afternoon levels could be explained by the increased concentrations of free plasma cortisone. This would lead to product inhibition in the conversion of cortisol to cortisone during its passage from plasma to saliva. The possibility of product inhibition by 11ßHSD by cortisone has been reported [26].
It would now be of interest to extend the current study to include salivary, total plasma and free plasma cortisol-cortisone measurements over a 24-h period in OC-users and pregnant women during gestation. This would determine whether in the night free plasma concentrations of cortisol and cortisone were normal and to what extent the raised total plasma and/or free plasma cortisol concentrations contribute to salivary cortisol levels.

It may be concluded from the results described here that there is a shift in the peak time of diurnal rhythms of salivary cortisol and cortisone towards later in the morning in conditions where CBG or total plasma cortisol are raised non-patholagically, i.e. OC usage or pregnancy. This process proceeds steadily during pregnancy fron the first trimester until term.
The concentrations of salivary and total plasma cortisol and cortisone are raised significantly during pregnancy. This results in increased S-AUC values for these two hormones in both plasma and saliva, whereas during OC usage only the total plasma concentrations of cortisol and the matched S-AUC values are increased significantly. A discrepancy was shown to exist between the daily rhythms of salivary and free plasma cortisol. The values for free plasma cortisol were normal during the afternoon but were elevated in saliva.
The observed time shift in the daily rhythms of cortisol and cortisone under conditions of raised CBG concentrations suggests that single time point comparisons of the concentration of these two hormones should be interpreted with caution.

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CHAPTER 7

The relation between cortisol and cortisone in saliva and in plasma in various hormonal conditions

The relation between cortisol and cortisone in saliva and in plasma in various hormonal conditions

INTRODUCTION

The possibility to measure cortisol in saliva as an indicator of stress has been known since the work of Shannon and coworkers [1-13]. Renewed interest in saliva as biological medium for clinical chemical purposes appeared with the development of more accurate and especially more sensitive methods, e.g. the immunoassay. Indications that salivary cortisol might replace the laborious measurement of plasma free cortisol led to a review of earlier data concerning levels of plasma free and salivary cortisol in several conditions [14,15].
It is expected that when total plasma cortisol is increased concomittant with Corticosteroid Binding Globulin (CBG), the plasma free level of cortisol, as a result of the negative feedback system of the Hypothalamo Pituitary Adrenal Axis (HPAA), will be in the normal range and symptoms of hypercortisolism will not be exhibited [16,17]. This increase in total cortisol together with CBG is found in pregnant women and women who take estrogen-containing oral contraceptives (OC's) [18-25]. Earlier data, however, are contradicting at the point of the level of plasma free cortisol in these conditions [26-52]. Several factors may underlie these contradicting results. They include methodological and physiological factors [53-63]. The main factors leading to erroneous and/or deviating results are dilution of sample without proper correction, and disregard of the daily rhythm of cortisol, respectively.
If salivary cortisol is to replace plasma free cortisol as a measure of adrenal activity certain conditions have to be met:
1) free diffusion from plasma to saliva;
2) independent on flow rate or diffusion rate;
3) no passage of binding protein;
4) no influence of metabolic reactions.

There is general agreement that the first two conditions are met. With regard to binding proteins, in this respect mainly CBG, its presence has been demonstrated in saliva but its origin is still a matter of controverse [64,65].
The activity of the enzyme 11ßHSD in the salivary gland is generally accepted; about half of plasma free cortisol is converted into cortisone, a presumed inactive metabolite [65-67]. 11ßHSD is present in virtually all tissues and it has a definite role in mineralocorticoid target organs and in placenta [68-76]. About its function in the salivary gland nothing is known, but it is said that its activity has no influence on the applicability of salivary cortisol [77]. In most studies salivary cortisol is measured with RIA, whether directly or after an extraction step, which is more or less aspecific due to cross-reacting compounds, including cortisone. Ferguson [78] already expressed his amazement about the fact that the proportionality of plasma free and salivary cortisol is maintained despite conversion.

This study was designed to investigate the relation between cortisol in saliva and free in plasma. Special care was taken to use the best available methods. Besides, to avoid any influence of the daily rhythm of corticosteroids, all samples were taken at about 9.00 h a.m. Another feature of the study was to investigate whether in conditions of raised CBG, i.e. during pregnancy or usage of OC's, levels of free cortisol in plasma indicate a state of hypercorticism and, if so, whether this is reflected in salivary cortisol levels. A further feature of the study was to investigate the influence of changed hormonal status on the activity of 11ßHSD in the salivary gland. Finally, another aspect of the study was to get more insight in the mechanism of passage of cortisol (and cortisone) from plasma to saliva, especially regarding the influence of 11ßHSD on the relation between plasma free and salivary concentrations.

MATERIALS AND METHODS

Subjects

Five groups of women were included in the study:
a) a control group (N-groep): 14 normal, healthy women not taking any medication;
b) a pregnant group (P-group): 15 pregnant women at the end of pregnancy;
c) a postpartum group (PP-groep): 18 women 6 weeks after delivery;
d) an OC group (M-group): 23 women taking Marvelon, (30 µg ethinyl estradiol + 150 µg desogestrel) for more than 3 months;
e) another OC group (S-group): 26 women taking Stediril, (30 µg ethinyl estradiol + 150 µg levonorgestrel) for more than 3 months).

All women were 18 - 45 years.

Sampling

Matched samples of blood and saliva werd collected at 9.00 - 9.30 h a.m. For the N-group and the OC-groups on day 18 of the menstrual and pill cycle, respectively. For the P-group at week 38 - 40 and for the PP-group at 6 weeks postpartum.

Blood was drawn into heparinized tubes, centrifuged and the plasma was stored at -20°C until analysis. Citric acid stimulated saliva was collected into plastic cups and frozen at -20°C. Before analysis these were thawed and centrifuged and the clear supernatant was used for the determinations.

CBG

Plasma CBG concentrations were determined with a CBG RIA kit purchased from Medgenix (Brussels, Belgium).

Plasma total and salivary cortisol and cortisone

Concentrations of plasma total and salivary cortisol and cortisone were measured as described earlier [79]. In short, to 0,2 ml plasma or 0,5-0,7 ml saliva were added tritium labeled

cortisol and cortisone as recovery tracer. The samples were extracted with methylenechloride and paper chromatographed in a descending Bush B5 system. Individual corticosteroids were analyzed with in-house RIA.

Plasma free cortisol and cortisone

Concentrations of plasma free cortisol and cortisone were determined with equilibrium dialysis followed by extraction, paper chromatography and RIA as described above. Details of this method are described earlier [79].

Statistics

Statistical evaluation of the results was performed with SAS Software (SAS Institute Inc., Cary, NC)

RESULTS

Comparison of groups

Mean values

For all five study groups the mean values (± S.D.) for each parameter were calculated. The results are shown in Table 1. Additionally, sum parameters for plasma free and salivary cortisol + cortisone were calculated for each subject and the mean values are included in Table 1.

It appeared that in the P-group all parameters were increased compared to the N-group. Salivary cortisol, however, did not reach statistical significance when calculated with ANOVA Bonferoni t-test. However, when applying Student's t-test, mean values were significantly different.

The PP-group was statistically comparable to the N-group. Mean free cortisol was elevated but individual values were in a broad range of levels. Again when using Student's t-test, mean plasma free cortisol was significantly elevated above normal in the PP-group. Both the M- and the S-group showed mean values between the N- and the P-group. CBG and cortisol levels approached pregnant values, but cortisone levels were nearer to the normal range.

Hypercortisolism

Plasma free cortisol is regarded to be the best parameter for the assessment of a condition of hypercortisolism. In normal condition plasma free cortisol ranges from 332 - 842 ng/dl. Mean values were significantly increased in the P-, the M- and the S-group. Individual values ranged from 905 - 1586 ng/dl, 756 - 1787 ng/dl and 366 - 1366 ng/dl, respectively. From these results if must be concluded that pregnancy and OC-usage lead to a state of hypercortisolism, although some overlap with normal values exists in OC-using women.

Table 1. CONCENTRATIONS OF CBG, CORTISOL AND CORTISONE

PARAMETER	NORMAL	(n)	POSTPARTUM	(n)	PREGNANT	(n)
CBG (mg/L)	46.8 ± 6.4[b]	(14)	45.5 ± 4.7[b]	(16)	94.0 ± 20.7[a]	(15)
TOTAL F (ng/dL)	13533 ± 2700[b]	(14)	18821 ± 588[3b]	(17)	35541 ± 8686[a]	(14)
FREE F (ng/dL)	594 ± 173[b]	(14)	817 ± 40[1b]	(18)	1122 ± 187[a]	(15)
SALIVARY F (ng/dL)	302 ± 78	(14)	386 ± 174[b]	(17)	525 ± 123	(15)
TOTAL E (ng/dL)	1838 ± 324[b]	(14)	2066 ± 567[b]	(18)	5393 ± 1039[a]	(15)
FREE E (ng/dL)	376 ± 80[b]	(14)	366 ± 91[b]	(18)	943 ± 209[a]	(15)
SALIVARY E (ng/dL)	834 ± 140[b]	(14)	876 ± 225[b]	(17)	1649 ± 271[a]	(15)
SALIVARY F+E (ng/dL)	1136 ± 176[3]	(14)	1262 ± 383[1]	(18)	2173 ± 332[2]	(15)
FREE F+E (ng/dL)	970 ± 224	(14)	1183 ± 463	(18)	2065 ± 289	(15)

PARAMETER	MARVELON	(n)	STEDIRIL	(n)
CBG (mg/L)	79.8 ± 14.8[ab]	(23)	75.6 ± 15.8[ab]	(25)
TOTAL F (ng/dL)	46544 ± 11422[ab]	(23)	38238 ± 9788[abc]	(23)
FREE F (ng/dL)	1170 ± 327[ab]	(23)	903 ± 321[ac]	(18)
SALIVARY F (ng/dL)	683 ± 251[ab]	(23)	468 ± 174[ac]	(26)
TOTAL E (ng/dL)	2121 ± 381[b]	(23)	2318 ± 402[ab]	(25)
FREE E (ng/dL)	306 ± 67[b]	(23)	319 ± 66[b]	(18)
SALIVARY E (ng/dL)	1322 ± 304[b]	(23)	1032 ± 29[4bc]	(26)
SALIVARY F+E (ng/dL)	2005 ± 523[4]	(23)	1500 ± 441[4]	(26)
FREE F+E (ng/dL)	1475 ± 361	(23)	1222 ± 341	(18)

Legend to Table 1: CBG = corticosteroid binding globulin; F = cortisol; E = cortisone; (n) = number of subjects; differences between groups are calculated with Anova multiple t-test; a = different from normal; b = different from pregnant; c = different from marvelon; differences between plasma free and salivary cortisol + cortisone are calculated with paired t-test; 1 = not significant; 2 = p<0.02; 3 = p<0.01; 4 = p<0.0001.

Table 2. CORRELATION BETWEEN PLASMA AND SALIVARY CORTISOL AND CORTISONE

REGRESSION Y vs X	NORMAL	N	POSTPARTUM	N	PREGNANT	N
SF vs FF	Y = 0.34 X + 101 R = 0.7522 P<0.002	13	Y = 0.32 X + 109 R = 0.7113 P<0.002	16	Y = 0.53 X - 70 R = 0.8029 P<0.0005	14
FF vs TF	Y = 0.05 X - 22 R = 0.7095 P<0.005	13	Y = 0.05 X - 64 R = 0.6655 P<0.005	16	Y = 0.006 X + 867 N.S.	13
SF vs TF	Y = 0.02 X + 74 R = 0.5846 P<0.05	13	Y = 0.01 X + 85 N.S.	15	Y = 0.003 X + 384 N.S.	13
SE vs FE	Y = 0.80 X + 536 N.S.	13	Y = 1.3 X + 381 R = 0.5334 P<0.05	16	Y = 0.5 X + 1187 N.S.	14
FE vs TE	Y = 0.14 X + 121 R = 0.5570 P<0.05	13	Y = 0.08 X + 209 R = 0.4739 P<0.05	17	Y = 0.13 X + 238 R = 0.6487 P<0.01	14
SE vs FE	Y = 0.24 X + 395 R = 0.5527 P<0.05	13	Y = 0.18 X + 499 N.S.	16	Y = 0.09 X + 1148 N.S.	14
SF vs FE	Y = 0.67 X + 50 R = 0.6919 P<0.01	13	Y = 0.95 X + 33 R = 0.4920 P<0.05	16	Y = 0.12 X + 408 N.S.	14
SE vs FF	Y = 0.11 X + 770 N.S.	13	Y = 0.40 X + 538 R = 0.6724 P<0.005	16	Y = 0.61 X + 960 N.S.	14
SFE vs FFE	Y = 0.46 X + 694 R = 0.5803 P<0.05	13	Y = 0.64 X + 481 R = 0.7331 P<0.001	16	Y = 0.80 X + 528 R = 0.6954 P<0.005	14
FFE vs TFE	Y = 0.05 X + 162 R = 0.6691 P<0.01	13	Y = 0.05 X + 164 R = 0.6380 P<0.01	16	Y = 0.005 X + 182 N.S.	13
SFE vs TFE	Y = 0.03 X + 614 R = 0.5502 P<0.05	13	Y = 0.03 X + 647 N.S.	15	Y = 0.05 X + 1921 N.S.	13

REGRESSION Y vs X	MARVELON	N	STEDIRIL	N
SF vs FF	Y = 0.62 X - 47 R = 0.8143 P<0.0001	22	Y = 0.49 X + 15 R = 0.9189 P<0.0001	17
FF vs TF	Y = 0.02 X + 160 R = 0.7573 P<0.0001	22	Y = 0.02 X + 198 R = 0.5473 P<0.02	17
SF vs TF	Y = 0.01 X + 59 R = 0.6113 P<0.002	22	Y = 0.01 X + 121 R = 0.5068 P<0.01	24
SE vs FE	Y = 2.2 X + 663 R = 0.4754 P<0.05	22	Y = 2.9 X + 122 R = 0.6193 P<0.01	17
FE vs TE	Y = 0.11 X + 79 R = 0.6062 P<0.005	22	Y = 0.06 X + 177 N.S.	17
SE vs FE	Y = 0.30 X + 692 N.S.	22	Y = 0.31 X + 331 R = 0.4136 P<0.05	24
SF vs FE	Y = 1.41 X + 254 R = 0.6919 P<0.01	22	Y = 1.03 X + 132 R = 0.4920 P<0.05	17
SE vs FF	Y = 0.71 X + 497 R = 0.7584 P<0.0001	22	Y = 0.63 X + 493 R = 0.6451 P<0.005	17
SFE vs FFE	Y = 1.22 X + 212 R = 0.8396 P<0.0001	22	Y = 1.14 X + 129 R = 0.8574 P<0.0001	17
FFE vs TFE	Y = 0.02 X + 376 R = 0.7215 P<0.0001	22	Y = 0.02 X + 463 R = 0.5333 P<0.05	17
SFE vs TFE	Y = 0.03 X + 579 R = 0.6465 P<0.001	22	Y = 0.02 X + 668 r = 0.4614 P<0.05	24

Legend to Table 2: Correlations between parameters were calculated with Pearson regression analysis; R = correlation coefficient; P = probability value; SF = salivary cortisol; FF = plasma free cortisol; TF = plasma total cortisol; SE = salivary cortisone; FE = plasma free cortisone; TE = plasma total cortisone; SFE = salivary cortisol + cortisone; FFE = plasma free cortisol + cortisone; TFE = plasma total cortisol + cortisone; N = number of subjects.

Table 3. PERCENTAGES OF PLASMA FREE AND SALIVARY CORTISOL AND CORTISONE

PARAMETER	NORMAL	POSTPARTUM	PREGNANT	MARVELON	STERIRIL
% FF	4.37 ± 0,97	4,20 ± 1,40	3,17 ± 0.65	2,54 ± 0.49	2,45 ± 0.72
(N) DIFFERENCE	(14) b,c	(17) b,c	(14) a,c	(23) a,b	(18) a,b
% FE	20.62 ± 4.09	18.24 ± 4.04	17.60 ± 2.81	14.53 ± 2.78	13.68 ± 2.74
(N) DIFFERENCE	(14) b,c	(18) a,c	(15) a,c	(23) a,b	(18) a,b
% SF	2,24 ± 0.46	1.93 ± 0.72	1.47 ± 0.37	1.48 ± 0.44	1.24 ± 0.40
(N) DIFFERENCE	(14) b,c	(16) b,c	(14) a	(23) a	(18) a
% SE	45.84 ± 7.09	43.41 ± 11.51	31.25 ± 6.14	63.52 ± 14.84	45.19 ± 11.79
(N) DIFFERENCE	(14) b,c	(17) b,c	(15) a,c	(23) a,b	(25) b,c
% FFE	6.33 ± 1,16	5.64 ± 1.40	5.13 ± 1.04	3.07 ± 0.58	3.15 ± 0.76
(N) DIFFERENCE	(14) b,c	(17) c	(14) a,c	(23) a,b	(18) a,b
% SFE	7.52 ± 1.20	5.86 ± 1.58	5.40 ± 1.16	4.18 ± 0.88	3.79 ± 1.03
(N) DIFFERENCE	(14) b,c	(16) a,c	(14) a,c	(23) a,b	(25) a,b

Legend to Table 3: For parameter definition see Table 2; (N) = number of subjects; DIFFERENCE = difference between study groups calculated with Anova multiple t-test; a, b, c = significantly different from normal, pregnant or marvelon, respectively.

Table 4. RATIOS OF PLASMA FREE AND SALIVARY CONCENTRATIONS OF CORTISOL AND CORTISONE

RATIO	NORMAL	POSTPARTUM	PREGNANT	MARVELON	STEDIRIL
FF/SF	1.98 ± 0.39	2.32 ± 0.82	2.20 ± 0.39	1.81 ± 0.42	1.98 ± 0.28
(N) DIFFERENCE	(14)	(17)	(15)	(23) c,d	(18) d
SE/FE	2.28 ± 0.45	2.42 ± 0,62	1.79 ± 0.30	4.44 ± 1.03	3.33 ± 0.85
(N) DIFFERENCE	(14) a,b,c	(17) a,b,d	(15) a,b,c	(23) b,c,d	(18) a,c,d
FF/FE	1.60 ± 0.46	2,19 ± 0.89	1.24 ± 0.29	3.91 ± 0.97	2.92 ± 1.24
(N) DIFFERENCE	(14) a,b	(18) a,b,d	(15) a,b,c	(23) b,c,d	(18) a,c,d
SF/SE	0.37 ± 0.09	0.43 ± 0.11	0.32 ± 0.07	0.51 ± 0.13	0.46 ± 0.14
(N) DIFFERENCE	(14) a,b	(17) a,d	(15) a,b,c	(23) c,d	(26) d
SFE/FFE	1.21 ± 0.24	1.09 ± 0.31	1.06 ± 0.12	1.37 ± 0.18	1.25 ± -.20
(N) DIFFERENCE	(14) a,1	(17) a,b,ns	(15) a,b,ns	(23) c,d,2	(18) 4
SE/FFE	0.90 ± 0.23	0.77 ± 0.23	0.80 ± 0.11	0.91 ± 0.13	0.88 ± 0.19
(N) DIFFERENCE	(14) ns	(17) a,1	(15) 2	(23) c,3	(18) 4
TFTE	7.46 ± 1.56	9.24 ± 2.46	6.77 ± 1.92	22.43 ± 5.82	16.74 ± 4.56
(N) DIFFERENCE	(14) a,b	(17) a,b	(14) a,b	(23) b,c,d	(25) a,c,d

Legend to Table 4: a = different from Marvelon; b = different from Stediril; c = different from postpartum; d = different from prganant; 1,2,3,4 = significantly different from 1.00, P < 0.001, 0.0001, 0.005 en 0.02 repectievely.

The postpartum group was sampled at six weeks after delivery, at which time hormonal status is generally believed to be returned to normal. With regard to the plasma free cortisol individual values ranged from 203 - 1659 ng/dl and eight out of eighteen values were above the normal maximum. This contradicts the general assumption mentioned above.

Salivary cortisol is considered to give a reflection of plasma free levels. The normal range was from 173 - 405 ng/dl. Comparing these values with those of the other study groups it appeared that in the pregnant group despite a raised mean value and the finding that only three out of 15 samples were within the normal range, statistically there was no significant difference between these two groups when calculated with ANOVA Bonferoni t-test, but when using the Student's t-test salivary cortisol is significantly elevated in pregnancy.
In the postpartum group six out of 18 samples were above the normal range. Salivary cortisol in both OC groups was significantly elevated; in the M-group only two samples were in the normal range and in the S-group only one sample.

Plasma free and salivary cortisone
Plasma free cortisone was significantly elevated above normal only in the pregnant group, a finding which was also true for salivary cortisone. Comparing both OC groups it appeared that mean salivary cortisone was significantly lower in the S-group than in the M-group.

Sum of cortisol + cortisone
The passage of corticosteroids from plasma to saliva is considered to proceed via a diffusion process. Metabolism in the salivary gland converts cortisol into cortisone. Hence, it was expected that levels of plasma free cortisol + cortisone (FFE) would not be different from salivary cortisol + cortisone (SFE). However, as must be concluded from the results in Table 1, SFE was significantly higher than FFE, except in the postpartum group. This contradicts a simple diffusion process.

Relations between several parameters
To determine the relations between the various parameters initially normal regression analysis was performed.

CBG and cortisol
Plasma total cortisol is expected to increase in concordance with CBG. A statistically significant correlation ($p<0.05$) between these two parameters was only found in the Marvelon and the postpartum groups, but did not exist in the other study groups. Any correlation between CBG and the other parameters measured could not be demonstrated.

Plasma free and salivary cortisol
Salivary cortisol was significantly correlated with plasma free cortisol in all study groups.

Plasma free and salivary cortisone
Salivary cortisone correlated significantly with plasma free cortisone in normal conditions and during OC usage, but not during pregnancy and at 6 weeks postpartum.

Plasma free and salivary cortisol + cortisone
The sumparameter for cortisol + cortisone showed significant correlations between plasma free and salivary concentrations in all study groups.

Percentage free and salivary cortisol and cortisone
Cortisol in plasma is for the greater part bound to CBG. As shown in Table 3, the fraction of free cortisol (%FF) accounts for about 4% of the total concentration in plasma in normal conditions and postpartum. This fraction is significantly lower at the end of pregnancy, due to the increase of CBG. In the OC groups the percentage free cortisol was even more decreased: 2,5%. The same holds true for the free fraction of cortisone (%FE) and also for the combined fractions of free cortisol + cortisone (%FFE).

The fraction of cortisol in saliva relative to the plasma total concentration is about half that of the plasma free fraction, due to metabolic conversion. This conversion seems to be greater in both OC-groups. This is reflected in the fraction of salivary cortisone relative to plasma total cortisone (%FE). The mean %FE in both OC-groups is more than 3 times the %FE, whereas in the other study groups this factor is slightly higher than 2.

The higher binding of corticosteroids in conditions of increased CBG levels is apparent when comparing the values of the fractions of plasma free cortisol + cortisone and salivary cortisol + cortisone.

Ratios of cortisol over cortisone

Plasma total cortisol and cortisone
For each subject the ratios of cortisol over cortisone were calculated and values expressed as mean levels per study group (Table 4). Cortisol is the principal corticosteroid hormone and cortisone is considered a biologically inactive metabolite. Degradation finds place mainly in the liver and in normal conditions the ratio of plasma total cortisol over cortisone (TF/TE) is about 7.5. In pregnancy the placenta is an additional source of cortisone, but it appeared that TF/TE was slightly lower than normal, although not significantly different. In the postpartum period TF/TE was slightly increased, but not different from mean normal or pregnant values.
During OC usage plasma total cortisol increases to about 3 times normal values, which is not the case with cortisone. As a result the ratio TF/TE is greatly increased in both OC-groups, the M-group being even significantly higher than the S-group. This may indicate a relatively lower metabolism of cortisol in the liver.

Plasma free cortisol and cortisone
The ratio of the plasma free concentrations (FF/FE), shown in Table 4, is in all study groups 4-5 times lower than TF/TE. This ratio of TF/TE is 1.60 in normal conditions, not signifi-

cantly different from pregnant and postpartum. In both OC-groups the ratio of FF/FE is significantly increased, again indicating relatively less conversion of cortisol into cortisone in the liver. In the M-group this ratio is significantly higher than in the S-groups, comparable to the ratios of TF/TE.

Salivary cortisol and cortisone
In saliva the ratio of cortisol over cortisone (SF/SE) in normal conditions is 0,37, being no different from pregnant values. In both OC-groups and in the postpartum group this ratio is significantly increased.

From these results it should be concluded that pregnancy has no influence on the proportion of F : E, whereas usage of OC's is a factor that inhibits conversion of F into E in the liver as well as in the salivary gland.

DISCUSSION

By using the methods described in this study several defects of earlier investigations could be avoided. Equilibrium dialysis of undiluted plasma samples combined with specific RIA of cortisol and cortisone in the dialysate provided reliable values for their plasma free concentrations. It appeared that in the pregnant and both OC groups mean plasma free cortisol was significantly higher than normal. The slight but not significant increase in the postpartum group was caused by a few women showing values above normal, indicating that at this time after pregnancy adrenal status was sometimes not yet normal. With regard to plasma free cortisone, only the pregnant group showed significantly elevated levels.
The best measure to demonstrate a state of hypercorticism is believed to be the level of plasma free cortisol. From the results of the present study it should be concluded that at the end of pregnancy and during OC usage women are in a state of hypercorticism. However, considering results published earlier with regard to the daily rhythm of cortisol in saliva, increased or decreased values of plasma free cortisol at one time point during the morning may be caused by a shift in the daily rhythm [80,81]. This may also apply to the women in the study described here, which means that the hypercortisolism found may be a false effect caused by said peakshift.

The determination of the plasma free fraction of steroid hormones, especially those that for the greater part are bound to high affinity binding proteins, is laborious, time consuming and prone to erroneous results. Already in the early sixties Shannon and coworkers [1-8] demonstrated a good correlation between salivary and plasma corticosteroids and they suggested that the latter may be replaced by the former. In the subsequent period, where methods were improved, it was demonstrated that salivary corticosteroids, particularly cortisol, was highly correlated with plasma free levels and was an even better measure for adrenocortical activity than plasma total cortisol [82]. In the present study, however, contradicting results were found. Although comparison of values of plasma free cortisol indicated a hypercorticism at the end of pregnancy and during OC-usage and despite good correlation

between plasma free and salivary cortisol, this was not reflected in salivary levels. It appeared that in this regard salivary cortisol was a less discriminating parameter.

The finding that the differences between subjects with normal or high CBG with regard to salivary cortisol are less than with regard to plasma free cortisol are a consequence of the enzymatic activity of 11ß-HSD in the salivary gland. About 30% of free cortisol is converted into cortisone and this percentage differs between groups. 11ß-HSD is an oxido-reductase which catalyzes the oxidation of cortisol to cortisone in the presence of NADP and the reduction of cortisone to cortisol in the presence of NADPH. The two activities are contained within one glycoprotein. Immunochemical analysis has demonstrated the presence in several tissues. However, each tissue preferentially exhibits only one activity: reductase or oxidase [83,84]. The direction of the enzymatic activity is dependent on the conditions present in the particular cells/tissues. The exception seems to be the placenta, where the 11ß-HSD activity gradually changes from predominantly reductase to predominantly oxidase activity. However, recently it was demonstrated that also the presence of an oligosaccharide component on the enzym dictates its activity in the direction of reductase or dehydrogenase. With regard to the salivary gland, it has been suggested that here only the dehydrogenase part of 11ß-HSD is active, with less than 1% reductase activity.

There are several hormonal factors that may influence the activity of 11ß-HSD. Data about this subject, however, are sometimes contrasting. Cortisol, a major substrate in human, exerts substrate inhibition at high concentrations [85]. On the other hand, it activates the enzyme by inducing enzyme synthesis, e.g. in fibroblasts [86].
Cortisone in relatively high concentrations exerts product inhibition, e.g. placenta [87,88], but in fibroblasts it stimulates dehydrogenation (F into E) [86]. With regard to progesterone, this was demonstrated to inhibit 11ß-HSD activity in placenta [87], although such effect was not found by other investigators [89].

Cortisone is generally believed to be a biologically inactive metabolite of cortisol, although the finding that in fibroblast cells cortisone more than cortisol can induce aromatase activity may contradict this assumption. In glucocorticoid (GC) target cells, however, cortisol exerts biological activity. Besides, the affinity of GC receptors for cortisone is less than for cortisol. Considering the fact that in these target cells 11ß-HSD is present, the biological effect of cortisol is regulated by this enzymatic activity. As a consequence, adrenocortical status will not only be related to absolute levels of cortisol in blood, but concurrently to 11ß-HSD activity. Possibly the ratio of cortisol:cortisone may give a better indication of adrenocortical or glucocorticoid status. The importance of cortisone levels in assessing glucocorticoid status has also been expressed by Mammami & Siiteri (1991) [86].

Cortisol is synthesized in the adrenals. Only part of the total amount released into blood will be available to target cells. On the one hand part of it is metabolized in the liver, about 10-15% to cortisone, and of the rest about 90% is bound to CBG and albumine. Supposed that 11ß-HSD activity is a co-determinant of adrenocortical status any effect hereon caused by pregnancy or OC-usage may be deduced from the change in cortisol:cortisone ratio determined for the free fraction of plasma and in saliva.

In normal conditions the ratio of plasma free cortisol to plasma free cortisone appears to be about 1.60, which corresponds to values found in literature. In pregnancy this ratio is slightly but not significantly lower. In both OC groups, in contrast, this ratio is significantly elevated above normal. From these data it should be concluded that during OC-usage, but not at the end of pregnancy relatively more cortisol can enter the target cells.

If all plasma free cortisone is derived from plasma free cortisol, the percentual conversion for the present study groups can be calculated:

$$\frac{\textit{plasma free cortisone}}{\textit{plasma free cortisol}} \times 100\ \%$$

For the respective groups this percentage presented as mean ±SD (group) was: 39,53 ± 6,70 % (N), 33.66 ± 9.48 % (pp), 45.47 ± 6.16 % (P), 21.24 ± 4.76 % (M), and 27.63 ± 7.57 % (S). It appeared that the highest percentage was found in the pregnant group, being significantly different from the postpartum, Marvelon and Stediril group. A disturbing point in this comparison is, however, that in pregnancy cortisone is additionally derived from the placenta. The lowest percentage was found in the Marvelon group, being significantly different from all other groups. This could mean that in the Marvelon group the enzyme 11ß-HSD is the least active.
These calculated percentages are different from a percentage as calculated from total concentrations of cortisol and cortisone. The percentage total cortisone with respect to total cortisol was: 12.20 ± 2.25 % (N), 10.37 ± 2.77 % (pp), 13.45 ±2.66 % (P), 4.54 ± 1.17 % (M), and 5.93 ± 1.29 % (S), respectively. Again the lowest percentage was found in the Marvelon group, although not significantly different for the Stediril group.

Provided that in the salivary gland only cortisol can be converted into cortisone and not the reverse, and plasma free cortisone diffuses unchanged into saliva, the total concentration of salivary cortisone is the sum of plasma free cortisone plus the part of plasma free cortisol that was metabolized. The conversion rate of plasma free cortisol can be calculated by applying the following formula:

$$\frac{\textit{salivary cortisone - plasma free cortisone}}{\textit{plasma free cortisol}} \times 100\ \%$$

For the present study groups this rate was 85.49 ± 38.08 % (N), 67.39 ± 35.34 % (pp), 63.09 ± 24.65 % (P), 88.53 ± 16.32 % (M) and 84.76 ± 25.98 % (S), respectively. No significant differences were found for these percentages between the study groups, probably due to the high variation. However, it is clear that the enzyme is much more active in the salivary gland than in the liver, especially in the normal group and when OCs are used. Also the data suggest that at the end of pregnancy the activity is lower than normal, which might have been a consequence of product inhibition by cortisone, but this does not hold true for the postpartum group.

The mechanism of the passage of cortisol and cortisone from plasma to saliva was another subject of the present investigation. Although it is generally believed that non-protein-bound corticosteroids freely diffuse through the salivary gland, our results contradict this assumption, because with the exception of the post-partum group, the sum of cortisol + cortisone in saliva was significantly higher than the sum of cortisol + cortisone free in plasma. There are several possibilities to explain this phenomenon:

1. in the salivary gland water is resorbed, resulting in a concentration of constituents and consequently elevation of corticosteroid levels;
2. proteins, in this case CBG and/or albumin pass from plasma to saliva by diffusion, taking along bound corticosteroids;
3. CBG and/or albumin pass by ultrafiltration, with the same effect as mentioned in 2;
4. corticosteroids are extracted from the blood by CBG present in the extravascular space as a result of sequestration, which leads to elevated binding of corticosteroids and release in saliva;
5. CBG receptors are present on the membrane of cells of the salivary gland, which bind and internalize CBG and corticosteroids bound to it;
6. CBG is present in the salivary gland cells due to *de novo* synthesis; in this case internal CBG acts as a "sink" for corticosteroids and keeps the gradient in the direction of the cells comparable to extravascular CBG as mentioned in part 4;
7. glucocorticoid receptors are present in the salivary cell cytosol, acting in the same way as internal CBG in part 6.
8. there is an unknown GC-binding protein present in the cell membranes of the salivary gland.

In many aspects, structurally as well as physiologically, the salivary gland is comparable to the kidney. Both are excretory organs where from the capillaries water and several blood constituents are collected in the ducts to form a primary secrete. During passage through the ducts the fluid is modified by reabsorption of e.g. sodium ions to form the secundary secrete. In the salivary ducts, in contrast to kidneys, no water molecules are reabsorbed from the ducts [90]. This means that the first possibility, concentration of fluid, is unlikely. Diffusion of molecules through the membranes is restricted to lipid soluble compounds. Corticosteroids but no proteins can enter the saliva by diffusion, excluding possibility 2. Between the acinar cells of the salivary gland ultrafiltration can take place, but only molecules with a molecular weight of less than approximately 300 dalton will enter the excretion fluid. CBG and albumin, however, are relatively large proteins, and it is unlikely that they pass the acinar cells by ultrafiltration. CBG and albumin have been determined in saliva in very low concentrations. At first these proteins were considered as blood contaminants. Chu & Ekins [65] found some relation with concentrations in blood, but until now only very few data are available about the presence of CBG and albumin in saliva in relation to blood levels to give any indication about their origin.
At many sites CBG is present in the extravascular space. In 1984 Stephenson et al. [91] found that in the inner medulla-papilla of the rat kidney CBG is accumulated to 4 times higher molar concentrations than in plasma. On the basis of their results they proposed a countercurrent exchange model for renewable extravascular sequestration of free corticos-

terone (the active corticosteroid in the rat). The function of this extravascular CBG was thought to be to capture corticosterone and to allow aldosterone to occupy the mineralocorticoid receptor. The developmental pattern of corticosteroid binding in renal papilla showed similarity to that of plasma CBG and the origin of the sequestered extravascular CBG appeared to be the plasma CBG. Any extravascularly bound corticosterone is released again into the recurrent vasculature. The salivary gland shows a similar countercurrent vasculature. Moreover, the salivary gland is also a target organ for aldosterone comparable to the kidney. In the light of these similarities between the salivary gland and the kidney it would not be surprising if in the former there would also be found a sequestration of CBG. As a consequence, cortisol, in humans, could be accumulated and subsequently excreted into saliva in higher amount than that expected from simple diffusion. Unfortunately, sequestration of CBG in the salivary gland has until now not been investigated.
De novo CBG synthesis in the salivary gland is another possibility that can lead to accumulation of cortisol in the cell and subsequent release into saliva. In some glucocortoid target cells CBG has been determined intracellularly [92,93]. For example, in uterus an increase in intracellular CBG levels could be induced by estrogens, comparable to liver synthesis. According to Siiteri [94], CBG synthesis and transport to the cell membrane provides specificity to the target cells. In salivary gland cells such CBG synthesis has hitherto not been demonstrated, which leaves this possibility as an explanation for our findings.
Cortisol accumulation in target cells may also procceed by the action of glucocorticoid or mineralocorticoid receptors. A prerequisite to fit in our model is that these receptors are cytosolic or anchored in the membrane to be able to trap cortisol from the plasma. The discussion about a cytosolic or nuclear site for intracellular receptors, however, is still going on. In a recent report about immunohistochemical determination of progesterone receptors in the salivary gland, [95] showed that at least these receptors are retained in the nucleus.
It is possible that for GC-receptors the regulation in target cells is different than for progesterone and estrogen receptors. In recent publication (Akner et al. 1991) [96] it was demonstrated that in human gingival fibroblasts GC-receptors are co-localized with extranuclear microtubuli, enabling the transport of GC through the cytoplasm to the nucleus.

The last possibility suggested above, an unknown GC-binding protein in target cell membranes, is supported by the results of Alléra & Wildt (1992a,b)[97,98]. These investigators demonstrated the presence of such a specific binding protein in vesicles of rat liver plasma membrane. It was excluded that the protein was CBG or GC-receptor. The affinity for corticosterone (in rats the counterpart of cortisol) was even higher than with CBG. It appeared that this protein, which they called GCC = glucocorticoid carrier, was a specific, high affinity binder, operating at physiological concentration of corticosterone in blood. Furthermore, influx as well as efflux of corticosterone was exhibited, with almost the same kinetic quality although efflux occured at a slow time-course. The conclusion of these investigators was that there is no passive diffusion of corticosterone but steroid transfer is a thermodynamically spontaneous and effective process whereby the steroid is transferred from CBG in the blood to the receptor inside the cell.

Summarizing the above, to explain our findings that the concentrations of salivary corticosteroids (cortisol + cortisone) are significantly higher than those in free in plasma, it is most probable that comparable to rat liver cells a membraneous protein called GCC, plays a role in the accumulation of corticosteroids in the salivary gland; the steroid is bound to this GCC, transferred through the cytoplasm via GC-receptors connected with microtubuli and released again via the countercurrent mechanism as comparable to that in the kidney or via GCC efflux.
Furthermore, the ratio of cortisol:cortisone in saliva as well as free in plasma in the OC groups is higher than that in the normal and pregnant groups. This indicates an influence of OCs on the enzyme activity of 11ß-HSD in the salivary gland as well as in the liver.

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CHAPTER 8

General discussion

GENERAL DISCUSSION

The objective of the present study was to investigate the applicability of the assay of steroids in saliva, particularly corticosteroids and progesterone, to assess on the basis of salivary values the hormonal status of individual subjects. The final purpose of this study was to demonstrate that saliva can be used as an alternative biological medium for collecting data concerning corticosteroid concentrations to be employed as a better measure for assessing the hormonal status of an individual than those from plasma. This latter consideration is based on the "Free Hormone Hypothesis" of Robbins & Rall (1957) [1]. In short, they pose that of a total amount of a particular hormone in blood only the free, non-protein-bound fraction is biologically active. Since the formulation of this hypothesis two hormones have attracted great interest, namely thyroxin, which was the model compound for the "Free Hormone Hypothesis", and cortisol. The interest for cortisol can be explained by the fact that a concentration divergent from normal values in blood leads to explicit clinical symptoms. The best-known example is Cushing's syndrome due to overproduction of cortisol [2,3]. As a result of the development of improved methods for the determination of the free fraction of cortisol in blood it was be clearly demonstrated that Cushing's syndrome is associated with elevated levels of plasma free cortisol [4,5]. This phenomenon is referred to as hypercorticism or hypercortisolism. The opposite, namely that increased plasma free cortisol levels are associated with Cushingoid symptoms, does not hold true. For example, it appeared that due to the usage of estrogens the total concentration of cortisol sometimes increased about 2-3 times, but Cushingoid symptoms were not observed. It should be noted, however, that the determination of free cortisol in plasma not always led to unambiguous results and conclusions [6-8]. The same phenomenon has been observed with pregnant women [9,10].

The discrepancies concerning concentrations of free cortisol in plasma could for the greater part be reduced to methodological aspects. Accordingly, when it was found that salivary cortisol was strongly related with plasma free cortisol [11], attention was directed toward saliva as an alternative biological medium. The underlying consideration was that cortisol in saliva could only be derived from the free fraction in blood because, in contrast to steroids, proteins cannot pass through membranes [12]. The (a)polarity of cortisol permits this compound to diffuse through the salivary gland. The mechanism of this passage has been extensively described [13]. It was expected that, when using saliva to measure cortisol concentrations, in conditions of elevated plasma total cortisol but without Cushingoid symptoms, levels of salivary cortisol would not be increased above normal. Unfortunately, literature data again showed no unambiguous results concerning salivary cortisol concentrations [14,15].

An important confounding factor is the presence of the enzyme 11ß-hydroxysteroid dehydrogenase (11ßHSD) in the salivary gland. The activity of this enzyme that converts cortisol into cortisone disturbs the relation between plasma free cortisol and salivary cortisol. The existence of 11ß-HSD in e.g. the salivary gland has been known for a long time and its activity has been mentioned to explain the lower concentration of cortisol in saliva than that free in plasma [16,17,18]. Unfortunately, the knowledge about the conversion of cortisol into cortison for the measurement of cortisol has hardly led to the selection of monospecific analytic methods for cortisol, that is methods without interference by cortisone or other substances [18]. Particularly in direct immunoassays or immunoassays including extraction the cross-reactivity of cortisone will strongly influence the results. In the case of specific methods the presence and concentration of cortisone has only been determined in singular cases [19,20]. Hence, nothing is known about any influence of 11ßHSD on the relation between plasma free and salivary cortisol. Besides, the influence of hormonal status on the activity of 11ßHSD with regard to salivary corticosteroids has not been subject of investigation.

The hitherto unsolved question whether in women using estrogens (in the form of oral contraceptives, OCs) or in pregnant women a state of hypercortisolism exists at the level of plasma free cortisol and the question whether any hypercortisolism could be confirmed by measuring cortisol in saliva by very specific methods, has led to the investigation described herein. Initially much effort was put into optimizing analytical methods in order to obtain reliable results. An extensive description of the applied methods to specifically measure cortisol with sufficient sensitivity in plasma (total and free) and in saliva is given in Chapter 2 [21]. Because cortisone was an important factor for the posed questions, this steroid was included in the study and has been measured with the same methods.

A further confounding factor may be the daily rhythm of cortisol. It has already been mentioned that differences in sampling time may prevent comparison of results [18]. For this reason in the present studies all samples were collected at the same time in the morning, i.e. at 9.00 h. In view of the objectives of this investigation the groups of subjects were carefully defined and selected. The first part of the study was directed to the effect of OCs on the parameters plasma total cortisol, plasma free cortisol, salivary cortisol, plasma total cortisone, plasma free cortisone, salivary cortisone, and CBG. Two groups of women were included in this study: normal women and women using oral contraceptives. The women in the OC group used only Marvelon® (30 µg ethinylestradiol + 150 µg desogestrel) for more than six months.

According to the "Free Hormone Hypothesis" any form of hypercortisolism was expected to be indicated by elevated concentrations of plasma free cortisol compared to normal values. The applicability of salivary cortisol as substitute for plasma free cortisol should appear, besides a good correlation between these two parameters, from a similar or even better discrimination between both study groups. From the results of the first part of the investigation it appeared that usage of OCs (Marvelon) led to the expected significant increase in CBG (from 892 ± 123 nmol/l, n=15, to 1534 ± 284 nmol/l, n=23), and plasma total cortisol

(from 394 ± 107 nmol/l to 1284 ± 315 nmol/l). Further, it appeared that plasma free cortisol had increased after usage of OCs from 18.1 ± 7.95 nmol/l to 32.3 ±9.03 nmol/l. A similar increase was observed for salivary cortisol (from 9.2 ± 3.88 nmol/l to 18.8 ± 6.92 nmol/l), the ratio of free cortisol/salivary cortisol being not significantly different (0.52 and 0.58, resp.). Regarding the finding that the correlation between plasma free and salivary cortisol was highly significant ($p<0.001$) it was concluded: a) that usage of Marvelon leads to a form of hypercorticolism at the level of plasma free cortisol; and b) that salivary cortisol gives the same information as plasma free cortisol. However, obvious symptoms of hypercortisolism (Cushingoid symptoms) were not shown by the OC-women.

Cortisone is regarded as a biologically inactive metabolite of cortisol. Plasma total concentration is about 12% (controles) to about 5% (OCs) of the total plasma cortisol concentration. In plasma, cortisone is bound to CBG similarly as cortisol but with lower affinity. It was found, that usage of OCs substantially had no effect on the total plasma concentration of cortisone. The increase in CBG resulted in a decrease of plasma free cortisone. This finding is in contrast to the observed increase in salivary cortisone. This discrepancy between plasma free and salivary cortisone can be explained by the activity of 11ßHSD in the salivary gland, by which part of plasma (free) cortisol is converted into cortisone and contributes to the overall concentration of cortisone in saliva. This also explains the much lower correlation between plasma free and salivary cortisone. The ratio salivary/plasma free cortisone after OC usage is about two times higher than normal, in contrast to the ratio for cortisol. It can be hypothesized that this is due to the fact that after use of OCs there is more free cortisol available in plasma for passage and conversion.

From these results it is evident that 11ß-HSD converts a considerable part of cortisol into cortisone during transport through the salivary gland, but apparently this has no influence on the potential applicability of salivary cortisol as a substitute for plasma free cortisol. The apparent hypercortisolism at the level of plasma free and salivary cortisol after OC usage, however, is not consistent with the absence of any observed Cushingoid symptoms. Absence of Cushingoid symptoms in the presence of continuously elevated concentrations of the biologically active (plasma free and optionally salivary) cortisol may be explained by a kind of "resetting" of the feed-back system of the HPAA together with a decreased sensitivity of target organs. The occurence of a resetting of the HPAA has been suggested previously [22]. Decreased sensitivity of target organs may occur when the availability of the glucocorticoid receptor (GCR) has diminished by depletion or by competition of other compounds. Depletion of cytoplasmic receptor can occur in situations of long term elevation of effector compounds [23,24]. The major competitor for the GCR is progesterone, which exhibits a similar affinity as cortisol and has been reported to compete with cortisol for receptor binding sites on target cells [25]. However, OC-usage leads to a considerable decrease of progesterone concentrations. Another possibility for competition is by the components of the OC composition. It has been demonstrated that the major metabolite of the progestagenic component of Marvelon, 3-keto-desogestrel, binds to the progesterone receptor [26,27] and to the glucocorticoid receptor with high affinity [28].

Another condition which has been subject of extensive studies related to saliva measurements is human pregnancy. Pregnancy is accompanied with increased levels of CBG, plasma total en plasma free cortisol, indicating a state of hypercortisolism. An important difference with OC-usage is that during pregnancy plasma progesterone as well as plasma cortisone increase considerably. With regard to the above discussion it seemed relevant to detemine the effect of pregnancy on plasma free and salivary levels of cortisol and optionally on the activity of 11ßHSD.
For this investigation samples (plasma and saliva) were collected from a group of 37 pregnant women four times during pregnancy. For non-pregnant control samples from the same group of women plasma and saliva were collected again at 6 weeks post partum. It is generally considered that at this time the hormonal status has returned to normal. The subjects were divided into 2 groups. In the one group sampling time was restricted to about 9.00 h to compare the results with those of the study concerning the effect of OCs.
According to literature data progesterone shows no daily rhythm in normal conditions (no OCs, not pregnant). With regard to progesterone during pregnancy there are indications that at the end of the third trimester a daily rhythm has evolved [29]. This daily rhythm appears the inverse of that of cortisol, i.e. in the evening higher values than in the morning. To demonstrate the existence of any daily rhythm of progesterone during pregnancy the second group of pregnant women was sampled at 16.00 h.

The results of this study have been published (Chapter 3) [30]. The most remarkable finding was the excellent correlation between plasma free and salivary progesterone, which has been demonstrated only once before [31]. Both for the group as a whole as for individual subjects this correlation was highly significant ($p<0.0001$ and $p<0.005$, resp.).
The concentration of progesterone in saliva was about 50% of the plasma free concentration. This finding seems similar to the difference between plasma free and salivary cortisol, where 11ßHSD is responsible for the decrease in salivary concentrations. Recently it was also demonstrated that in the salivary gland of healthy individuals progesterone is metabolized to various metabolites [32]. Another cause of the "disappearance" of progesterone could be adsorption in the sampling device. It is kwown that progesterone strongly adsorbs onto several matrices. For this study saliva was collected in plastic cups. The samples were frozen and centrifuged before use. It was demonstrated that this method yields lower values than when samples are collected in glass tubes and sonificated before use. [Chapter 4, 33]. Adsorption to the wall of the plastic cup as well as onto particulate matter may cause the loss of progesterone in the saliva samples. Together with some metabolism in the salivary gland this may account for salivary levels being about half those free in plasma. Apparently the method of processing the salivary samples has no influence on the correlation between plasma free and salivary progesterone.

With regard to the question whether salivary progesterone corresponds with plasma free progesterone, the highly significant correlation between these two parameters is a positive indication herefore. Moreover, the increase in plasma free progesterone during pregnancy and the decrease postpartum is reflected in saliva. The question whether salivary progesterone can substitute plasma free determinations can not be answered straightforward and

depends on the objective of the measurements. Saliva seems excellently suitable for monitoring studies. On the other hand, comparing morning and afternoon values the discriminating capacity of salivary progesterone apparently is less than that of plasma free progesterone. The significant differences between plasma free progesterone in the morning and in the afternoon are less obvious in saliva.
Apparently a daily rhythm of progesterone with significantly higher levels in the morning could be demonstrated only at the level of free plasma concentrations, whereas in saliva the same trend was visible but differences were not statistically significant. It should be noted that at the end of pregnancy plasma total progesterone also exhibited a daily rhythm, but in reverse direction, i.e. higher values in the afternoon and evening than in the morning. This finding corresponds with values found in the literature [29,34]. The discrepancy between the daily rhythm in plasma total and plasma free progesterone can be explained by the competition between progesterone and cortisol for binding places on CBG. In the morning the concentration of cortisol increases above the capacity of CBG, displacing any progesterone and resulting in an increase of plasma free progesterone. This latter fraction will then be metabolized at a higher rate, resulting in a lowering of the total concentration. In the afternoon the reverse will take place.

In the same study group cortisol and cortisone were measured. The results are given in Chapter 5 [35]. With regard to plasma total cortisol and cortisone the expected increase in concentrations during pregnancy was observed in both morning and afternoon samples, with a significant decrease at 6 weeks postpartum. Plasma free cortisol levels in the morning showed similar development as plasma total concentrations. Compared to postpartum values, especially those in the second half of pregnancy were significantly elevated. Since in that period plasma free cortisone is also increased, it should be concluded that then a condition of hypercorticism exists.
Afternoon values of plasma free cortisol tend to increase in the course of pregnancy, but no statistically significant differences were found between the sampling times. That means that if cortisol should be measured only in the afternoon a condition of hypercortisolism would not have been detected. In contrast, plasma free cortisone concentrations are raised significantly in the morning as well as in the afternoon in the second half of pregnancy.
Measuring cortisol and cortisone in saliva both morning and afternoon values indicate a state of hypercorticism. As a consequence of the discrepancy between morning and afternoon values the question whether pregnancy is associated with a state of hypercortisolism can not be answered unambiguously. Considering cortisol as the major corticosteroid hormone being biologically active, the following questions remain to be answered:
1) Which time is relevant for sampling?
2) Which parameter is a measure for hypercorticolism, plasma free cortisol or salivary cortisol?
3) Which of these two parameters is a measure for biological availibility of cortisol?

For an answer to these questions reference is made to the study concerning the daily rhythm of corticosteroids as described in Chapter 6 [36]. In this study again three groups of women were included. Plasma was sampled during daytime (7.00 -16.00 h) and saliva was collec-

ted from early morning until late evening (6.00 - 24.00 h). It appeared that when taking mean values the peak in the daily rhythm for salivary cortisol and cortisone in the control group was 7.10 h en 7.26 h, respectively. During OC usage and at the end of pregnancy this peak was shifted to about 1-2 hours later in the morning. The course of the levels for salivary cortisol and cortisone were highly comparable. Unfortunately, the sampling of plasma started at 7.00 h and consequently the peak was missed and only the decrease of the peak in the daily rhythm could be observed. Plasma total cortisone showed only slight changes in concentrations. Plasma free cortisol and salivary cortisol behaved parallel. In contrast, the daily rhythm of salivary cortisone runs parallel to that of cortisol, whereas plasma free cortisone hardly shows any rhythm.
From the above it is evident that single-point measurements between 7 h and 10 h in the morning as have generally been employed in studies concerning the applicability of salivary cortisol [37,38], greatly influence the results. It is recommended to collect samples in the afternoon, especially when normal subjects are to be compared with those using OC's or pregnant.
Remains the question whether to use salivary cortisol or plasma free cortisol as a measure for hypercortisolism. Unfortunately, in the afternoon there exists a discrepancy between plasma free and salivary cortisol. Whereas for plasma free cortisol there is no difference between the three study groups, salivary cortisol is increased in the OC group and the pregnant group of women. Which of these 2 parameters is superior for indicating a state of hypercortisolism (see question 2 above) remains difficult to answer. Considering plasma free levels as the biologically active fractions, the conclusion has to be that during OC-usage and at the end of pregnancy there exists no state of hypercortisolism.
The measurement of salivary corticosteroids was intended to allow for replacement of the laborious measurement of plasma free levels. Given the results mentioned above, saliva does not entirely reflect plasma free levels. A confounding factor is the observation that absolute values of salivary cortisol are not similar to plasma free levels. Part of the plasma free fraction is converted into cortisone by 11ßHSD in the salivary gland and regarding the differences between the study groups the enzymatic activity may vary depending on hormonal status.

Besides the search for a reliable parameter for hypercortisolism, it was also to be assessed which fraction - plasma free or salivary cortisol - is a measure for biological activity. The presence and activity of the enzyme 11ß-HSD has been determined in corticosteroid target cells, e.g. in the salivary gland [39,40]. Due to the action of 11ßHSD the concentration of salivary cortisol is about half that free in plasma, the other part being converted into cortisone. Moreover, as mentioned above the extent of enzymatic activity may vary depending on hormonal status. To circumvent the influence of 11ßHSD, cortisone may be included in the calculations. According to the "Free Hormone Hypothesis" and assuming a free diffusion of corticosteroids from plasma to saliva, levels of salivary cortisol + cortisone should be similar to that free in plasma regardless of the presence of 11ßHSD.

To examine this issue from five groups of women (normal women, women at the end of pregancy and 6 weeks postpartum, women using Marvelon or Stediryl as an oral contracep-

tive) plasma and saliva were collected at 9.00 h a.m. and the concentrations of plasma free cortisone were compared with the corresponding salivary cortisol + cortisone. The results of the measurements and calculations are given in Chapter 7. It appeared that, with the exception of the postpartum group all women had significantly higher values for salivary cortisol + cortisone than for plasma free cortisol + cortisone. These results indicate that either the "Free Hormone Hypothesis" does not apply to corticosteroids or that passage of these steroids does not proceed through free diffusion. This implicates that the mechanism of transport of corticosteroids from blood to saliva has to be revisioned.

Scrutinizing the most recent literature not one but several modes of transport were found possible. These are summarized in the figure shown and will be explained below.
In the figure the different levels of transport are divided into entry, movement and processing in the cell, and excretion.

Part 1: Cortisol in plasma

In plasma cortisol exists in three different states: (**1a**) presents plasma free cortisol, up to about 4 %; (**1b**) presents albumine-bound cortisol, up to about 6 %; and (**1c**) presents CBG-bound cortisol, up to about 90 %. Cortisone is not given in the figure for reasons of clarity, but herefor the same distribution applies with percentage of 16, 6 and 39%, respectively [41].

Part 2: Entry into the cell:

Cortisol is a relatively small apolar compound and for a long time simple diffusion, (**2a**), through the membrane was considered the only way of entry into cells [42]. The same applies to cortisone. Any diffusion proces, however, implies transfer along a concentration gradient and, consequently, concentrations of cortisol + cortisone in saliva can never be higher than those free in plasma. It is improbable that this mechanism is the only driving force in transport.
Since many years a discussion has been going on between several working groups whether and to what extent albumine-bound and/or CBG-bound cortisol may form an additional source of cortisol supply [43-51]. Still, a subsequent delivery of cortisol from these two proteins in plasma could not drive the transfer of cortisol against a concentration gradient.

The second mechanism (**2b**) is through pinocytosis, the ingestion of fluid and solutes via small vesicles [52], allowing rapid accumulation of ingested solutes. Acceptor sites at the cell membrane are the clathrin-coated pits.

Endocytosis is also a mechanism by which CBG-bound cortisol may enter the cell (**2c**). Internalization of protein-bound steroids has been described [53,54], but is related to a specific receptor and is very low. This mechanism has been described for several steroid binding proteins such as sex hormone binding globuline (SHBG), corticosteroid binding globulin (CBG) and aldosterone binding globulin (ABG). The mechanism of uptake of cortisol through a CBG-receptor is further explained in part (**2e**). Simple endocytosis of CBG-bound cortisol will probably not take place.

Whereas the first three mechanisms described above are in principle non-specific for cortisol and CBG, transport of cortisol may take place via binding to specific acceptor molecules. The first candidate is the glucocorticoid receptor (GCR). This mechanism is presented as (**2d**).
Mineralocorticoid target organs, including the salivary gland [55-57] contain glucocorticoid receptors (GCRs) type I and II. The precise localization of receptors in a cell is still subject of debate. Although generally it is believed that GCRs are present in the cytoplasm, aldosterone receptors (GCR type I) have been demonstrated to be present in the membrane of monocytes and mononuclear leukocytes [58,59]. The presence of GCR type II (for glucocorticoids) in the cell membrane has been demonstrated in the nerve system of amphibian species and is suggested for other cell systems [58]. Thus, specific binding of cortisol to GCR localized in the membrane is possible, although not yet demonstrated for the salivary gland. The mechanism of the coated pits (see above), that are known to contain various receptors, e.g. for cholesterol [52], may also apply to steroid receptors in target cells.
Another receptor-mediated entry is via the CBG-receptor (**2e**). In many human tissues specific binding of CBG to the cell membrane of target tissues has been demonstrated [53,54,60-64]. Similar to binding to the GCR, cortisol could be bound to CBG-receptor at coated pits and thus be internalized into the cell. With regard to the concomitant uptake of steroids, e.g. cortisol, there are several reports that bound steroids enhance the binding of CBG to its receptors [53,60,62,64], although corticosteroids seem to inhibit the binding of CBG to rat liver membrane CBG receptors [65].

In (**2f**) the last mechanism is presented, namely the binding to the glucocorticoid carrier (GCC). Allera et al. [66,67] described the specific uptake of corticosterone in rat liver membrane vesicles via GCC. It appeared that GCC is a thermolabile specific binding protein with high affinity for corticosteroids and the ability to accumulate steroids in the cytoplasm. The affinity of GCC for cortisol was determined to be higher than to CBG. The conclusion of these authors was that uptake of corticosteroids in the rat liver cell is not a question of passive diffusion.

Part 3: Release into the cell/cytoplasm:

As indicated with (**3a/b**), cortisol derived from plasma free, albumine-bound or CBG-bound cortisol can easily diffuse through the membrane and is then released as either a free moving compound in the cytoplasm (**3a**) or bound to cytoplasmic components. It has been demonstrated on the basis of the determination of intracellular mRNA analysis that CBG is not only synthesized in the liver where it is released in blood as transport protein, but also in other tissues, especially mineralocorticoid target tissues [68-70]. A CBG-like molecule additional to the glucocorticoid receptor has also been demonstrated in the cytosol of inflammatory tissues [71]. The high affinity of CBG ensures a rapid binding of entering cortisol (**3b**).

The major binding component in corticosteroid target cells is the GCR (**3c**). Experiments carried out by Akner et al. [72] with antibodies agains GCR and microtubulin in gingival fibroblasts showed that GCR is cytoplasmatic and associated with microtubuli. Cortisol

binds to the GCR and after intracellular formation of a complex with various proteins, including heat shock proteins resulting in a so-called transportosome, this complex is transported via the cytoskelet transportsystem [73-75] to the nucleus.

Cortisol and CBG-bound cortisol taken up by pinocytosis may uneventfully be transferred through the cytoplasm (**3d**). As discussed above (**2c**), the internalization of CBG, with or without cortisol, has been demonstrated, but occurs probably via specific CBG receptors.

In the case of binding of cortisol to membrane-bound GCR (**3c**) in a target cell, a conformational change might release the GCR-cortisol complex into the cell, where it is further complexed into a transportosome in the same way as mentioned above. Another way of uptake and release is through endocytosis/internalization.

Similarly, specific binding of CBG to a high affinity binding protein has been demonstrated (see **2e**). The major function of the formation of a CBG-receptor complex is still a matter of debate. On the one hand, complex formation leads to the generation of second messenger (cAMP). On the other hand it can be internalized (**3e**) and is regarded as a transport mechanism to guide CBG to the nucleus [53,54,60,65,68]. However, the rate of internalization is very small (about 3 %). The function of cortisol in this mechanism seems to be the enhancement of the binding. It is unclear whether CBG or CBG-cortisol is released upon internalization.

In (**3f**) is depicted the release of cortisol from GCC. According to Alléra & Wildt [66,67], binding of cortisol to GCC induces a thermodynamically driven transfer of corticosterone in the case of rat hepatocytes from CBG in the blood to the receptor inside the cell. Entry proceeds by conformational changes, leading to a lowering of affinity and delivering to cytoplasmatic GCR. Presumably, the same mechanism occurs with cortisol in humans.

Part 4: Fate of cortisol inside the cell

Once cortisol is in the cytoplasm there are three destinations. When it is taken up in the microsomes (**4a**), either from the free state or internalized via vesicles, cortisol is metabolized by 11ßHSD and converted into cortisone. In the salivary gland this enzymatic activity occurs to such an extent that about half of the concentration of cortisol is converted into cortisone.

Unmetabolized cortisol may move to the opposite site of the cell (**4b**).

Receptor-bound cortisol, as is the case in most glucocorticoid target cells, is transported to the nucleus, where cortisol exerts its biological activity (**4c**) by binding to the corresponding reponse element. This mechanism has been described extensively before [76,77]. The receptor is recycled to the cytoplasm where it is degraded or used again for uptake of cortisol. Cortisol itself is released into the cytoplasm and metabolized or ex/secreted.

Part 5: Excretion of cortisol

If diffusion is a process of uptake of cortisol, excretion will take place via the same process for the same reasons (**5a**). Again, such a diffusion process will proceed along a concentration gradient. The same holds true for any cortisone, whether taken up in the same way as cortisol by diffusion or after conversion.

Active transport at the luminal site of the salivary cell from cytoplasma to saliva may occur against a concentration gradient if the mechanism involving the P-glycoprotein applies [78-80]. It has been demonstrated that in the membrane of secretion/excretion organs at the luminal side of the cells a particular glycoprotein, P-glycoprotein, is present which apparently binds and actively transports cortisol, cortisone and aldosterone from within the cell to the corresponding lumen. In contrast, progesterone is bound to the P-glycoprotein, but is not transported. The active transport mentioned above is energy dependent and can proceed against a concentration gradient resulting in accumulation in the lumen. Although P-glycoprotein has not yet specifically been demonstrated in the salivary gland, its presence in various transport epithelia makes it very plausible that it will be present here too.

Cortisol, whether or not bound to CBG, that is taken up by pinocytosis can be excreted in the same way (**5c, 5d**). Pinocytosis is a mechanism consisting of both endocytosis and exocytosis. Cortisol, cortisone and CBG-bound cortisol can be released at the luminal site of the salivary cell [52].

As is evident from the above, transport of cortisol from plasma to saliva may proceed via many steps. Diffusion is generally accepted as the process by which corticosteroids pass from plasma to saliva, but if this were the sole mechanism it could never explain why concentrations of cortisol + cortisone in saliva are significantly higher than those free in plasma. Whether pinocytosis occurs in the salivary cell is unknown. If this process takes place, it is more probable that it is associated with membrane-bound receptor for cortisol and/or CBG localized in clathrine-coated pits.
The most acceptable explanation for transport of corticosteroids is the existence of the carrier, GCC. As mentioned above, the affinity of cortisol for the GCC is higher than for CBG and as a result cortisol will be captured by the GCC from CBG. Further, the uptake is selective and active, so that cortisol can be accumulated in the cytoplasm. The presence of GCC in human cells, however, has to be demonstrated.
To explain the finding of increased salivary levels of corticosteroids in comparison with plasma free levels, excretion at the luminal site of the salivary cell must also proceed against a concentration gradient. Diffusion and exocytosis alone will probably not meet this condition. Here the P-glycoprotein presents a candidate mechanism of excretion. Both cortisol and cortisone are actively transported from the inside to the outside of the cell. Furthermore, the finding that during pregnancy only in the first months salivary cortisol + cortisone are increased above plasma free cortisol + cortisone can be explained by progesterone inhibiting the transport of cortisol. The results in OC-using women, where the difference between salivary cortisol + cortisone is the highest compared with normal and pregnant women, support this hypothesis because during use of OCs progesterone is suppressed.

The explanation given above implies that CBG-bound cortisol may additionally deliver cortisol to target organs and that the free fraction not exclusively determines the biological activity of cortisol. Remains the question how it can be explained that in conditions of raised plasma total cortisol and CBG, such as in pregnancy or during OC usage, there are no clinical symptoms of hypercortisolism. Maybe in these situations the activity of 11ßHSD plays an important role in inactivating cortisol inside the cells. Further explanations are a resetting of the setpoint of the feedback system of the HPAA, a decrease in the sensitivity of the adrenal for ACTH, and a refractoriness of target tissues [81].

As mentioned above one of the objects of this study was to get an indication about a possible influence of the activity of 11ßHSD on salivary corticosteroids in various conditions. Unfortunately, the presence of P-glycoprotein hampers any conclusion as to the activity of 11ß-HSD and even if this transportprotein really functions nothing is known about any difference in efficiency of transport of cortisol and cortisone. The transport of metabolites of steroid hormones by P-glycoprotein is at the moment under investigation [78].
Assuming that cortisol and cortisone show a comparable affinity for P-glycoprotein and are transported with similar efficiency, the differences in the ratios of the concentrations in saliva and free in plasma may provide an indication for the activity of 11ßHSD. There appeared to be no significant difference in the ratio of free cortisol/salivary cortisol, free cortisone/salivary cortisone, free cortisol/free cortisone, salivary cortisol/salivary cortisone and total plasma cortisol/total plasma cortisone between normal and pregnant women. This means, that 11ßHSD activity is similar in these conditions without substrate or product inhibition or inhibition by progesterone. Both OC groups, however, differ from the normal and pregnant groups in some respects. Compared to normal women the OC women have relatively more cortisone in saliva in relation to plasma free cortisone (ratio salivary cortisone/free cortisone). Considering that salivary cortisone is for the greater part derived from plasma free cortisol, and that plasma free cortisol is increased above normal after OC usage, this finding may indicate that the activity of 11ßHSD is increased during OC usage, which is conform data about enzym activation in the liver by OCs [82-86]. At least this conclusion applies for cortisol - cortisone dynamics in the morning. It is possible that after the morning peak of cortisol the situation is different. Solution of this problem awaits further study.

Summarized it can be concluded:

(1) That passage of corticosteroids from plasma through the salivary gland into saliva partly is an active energy-dependent process, involving transport of cortisol and cortisone against a concentration gradient resulting in salivary levels being higher than those free in plasma.
(2) That despite extensive metabolism salivary cortisol is significantly correlated with plasma free cortisol. Thus, monitoring of cortisol can be performed on the basis of salivary determinations.
(3) That for the assessment of hypercortisolism, however, salivary cortisol is less suitable. Salivary concentrations do not exactly reflect plasma free concentrations and the discriminating potency appears less than for plasma free cortisol as demonstrated in the

groups studied. Considering plasma free cortisol as a defined parameter of hypercortisolism saliva is not an alternative biological medium.

(4) That the data concerning the daily rhythm of plasma free cortisol indicate that at the end of pregnancy a form of hypercortisolism exists. In contrast, usage of OC does not lead to higher levels of plasma free cortisol. Most remarkable finding was a shift in peak time for cortisol in both pregnant and OC using women. Single-time point measurement thus may readily lead to erroneous conclusions as a consequence of this shift in the peak to later in the morning.

(5) That with regard to the daily rhythm of cortisol saliva again does not always reflect plasma free cortisol. In contrast to normal levels of plasma free cortisol in OC using women over the day, cortisol levels in saliva were increased. (6) That when plasma free cortisol defines its biological activity, salivary cortisol can not be used as an alternative. On the other hand, when the activity of 11ß-HSD is a measure of the biological activity of cortisol, salivary cortisol + cortisone may present a better parameter than plasma free cortisol alone. Unfortunately, conclusions of the activity of 11ß-HSD can not be given.

The results of this study strongly indicate that passage of cortisol from plasma to saliva is not merely passive diffusion, but rather at least partly a specific energy dependent process. Both uptake at the vascular site and release at the luminal site probably proceed through specific binding proteins against a concentration gradient. This phenomenon could explain the finding that concentrations of the corticosteroids cortisol + cortisone are higher in saliva than could be expected on the basis of plasma free levels. Furthermore, not only free cortisol is taken up and transported. The existence of a GCC protein at the vascular site of a salivary gland cell with an affinity for cortisol higher than that of CBG consequently leads to the conclusion that also CBG-bound cortisol may enter the cell.

Further Research

The first objective of this study was to determine whether saliva can be used as an alternative biological medium to assess the hormonal status of humans with regard to corticosteroids. The answer has to be no. It remains a matter of debate which parameter, plasma free or salivary cortisol, is a measure for hypercortisolism. Data after OC usage or in pregnancy compared with those of normal women are conflicting. The experiments should be extended with samples collected all over the day for 24 hours. Then plasma free and salivary cortisol and cortisone should be measured. The area under the curve may be a better measure to determine a state of hypercortisolism in certain conditions in relation to normal values.

The second objective was to deduce the activity of 11ßHSD as well as any change in enzymatic activity in several conditions on the basis of values of cortisol and cortisone in plasma and saliva. Initially, the basic assumption was that corticosteroids pass from plasma to saliva by simple diffusion. The results presented, however, contradict this assumption and passage seems to involve an active process. Consequently, too many confounding factors

prevent conclusions about this subject. Maybe it would contribute to our understanding of the transport of corticosteroids through the salivary gland if an extensive study on the activity of 11ß-HSD in the salivary gland could be performed with regard to several hormonal conditions. In addition, the half-life of 11ß-HSD may be relevant with regard to its activity over the day.

Whether protein-bound plasma corticosteroids contribute to levels found in saliva also remains a matter of debate. For example, the ratio of CBG/free plasma cortisol/GCC and possibly the influence of hormonal status on this ratio may add to explain varying concentrations in saliva. More research is needed to solve this problem. Investigation should relate to 1) uptake in the cells, and 2) secretion/excretion at the luminal side of the salivary gland. With regard to item 1) the question is what uptake processes take place at the plasma side. These may involve one or more mechanisms involving diffusion, membrane-bound carrier proteins or membrane-bound receptors. Similarly, the presence of P-glycoprotein at the luminal site of the salivary gland should be assessed and its action with regard to corticosteroids determined.

Another interesting feature is the influence of progesterone in the above mentioned mechanisms. It is not known how progesterone passes from blood to saliva and what mechanisms are involved. Because the sampling methods used precluded correct values for progesterone in saliva, in further studies these methods have to be optimized. This will permit conclusions about what fraction of plasma free progesterone will ultimately be found in saliva. Relating concentrations of cortisol, cortisone, progesterone and any major metabolite of progesterone may give further insight into any metabolism in the salivary gland and interference during uptake and/or secretion/excretion of the steroids.

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CHAPTER 9

Summary

SUMMARY

Chapter 1: Introduction

In Chapter 1 is explained in which form cortisol appears in plasma, i.e. with high affinity bound to CBG (corticosteroid-binding globulin), with lower affinity to albumin and as an unbound, free hormone. Generally, the free form is considered the biologically active component and an increase in the concentration of free cortisol is associated with severe clinically demonstrable abnormalities. A change in this concentration, however, can not be deduced from a total plasma concentration of cortisol due to the binding proteins and the action of the negative feed-back system. Accordingly, much attention has been given to the relation between total and free plasma cortisol on the one side, and to the measurement of free cortisol in plasma on the other hand. The fact is, that the latter has led to contradictory results, which can be attributed to the analytical methods used.

The demonstration that cortisol can be measured in saliva as well and that salivary values form a good reflection of plasma free cortisol, has stimulated a whole series of investigations. The final conclusion was, in short, that cortisol in saliva is derived from the free fraction in plasma by simple diffusion and therefore represents a good parameter for its biological activity.

The study described in this thesis was directed at the relation between free cortisol in plasma and salivary cortisol in conditions where the total concentration of cortisol in plasma is markedly increased with respect to normal, whereas this is not associated with symptoms, that indicate an increase in plasma free cortisol. By way of introduction an extensive literature study was conducted regarding this subject, the results of which are summarized in Table 1 and 2.

Additionally, literature data with regard to plasma free progesterone and salivary progesterone as well as data referring to the presence and the activity of the enzyme 11ß-HSD (11-beta-hydroxysteroid dehydrogenase) are given in Table 3, 4 and 5 respectively. Progesterone was included in the study because this hormone may influence the binding of cortisol to plasma proteins and consequently, due to displacement, the concentration of plasma free cortisol. 11ß-HSD was included in the study because this enzyme is present in the salivary gland, here converts cortisol into cortisone and consequently affects the concentrations of salivary cortisol and cortisone.

Chapter 2: The effect of oral contraceptives on plasma free and salivary cortisol.

Chapter 2 describes the results of an investigation regarding the effects of oral contraceptives (OCs) on the concentrations of plasma CBG, plasma total and free cortisol, plasma total and free cortisone, and salivary cortisol and cortisone.
It is recognized that due to the estrogenic component in OCs the concentration of CBG in plasma increases. This results in a concomitant increase in plasma total cortisol whereas in situations of OC usage no symptoms of pathologically increased cortisol are observed. The expectation that in such cases free cortisol will be in the normal range could not be demonstrated in earlier studies.
In the study described here the parameters mentioned above were measured with highly accurate methods to ascertain whether free cortisol in plasma of OC using women was increased with respect to normal values (control group). Additionally, it was ascertained whether values found for free cortisol in plasma were comparable to those in saliva. Moreover, with regard to the known activity of 11ß-HSD in the salivary gland cortisone was also measured in the same plasma and saliva samples.

Chapter 3: Salivary progesterone excellently reflects free and total progesterone in plasma during pregnancy.

In Chapter 3 the investigation regarding the relation between plasma total and free progesterone and salivary progesterone during pregnancy is described. Progesterone is a hormone, the concentration of which increases considerably in the course of pregnancy. This steroid hormone shows much similarity to cortisol with regard to binding to plasma proteins and passage from plasma to saliva. This was the reason to investigate how far salivary values of progesterone correspond with free plasma values.
For this study plasma and saliva samples were collected from pregnant women at 4 time points during pregnancy and as a control at 6 weeks postpartum. To be able to measure the above mentioned parameters analytical methods were adapted, improved and developed in order to obtain accurate results.

Chapter 4: The effect of pretreatment of saliva on steroid hormone concentrations.

In Chapter 4 the results of an investigation concerning the effect of pretreatment of saliva samples on the final results of steroid analyses are given. A standard method for collecting and processing saliva prior to analysis is the collection of saliva in plastic cups, freezing and thawing the samples to make them less viscous, centrifugation of the samples in plastic tubes to remove solid particles. Supernatant fluid is then used for measurements and consists of clear, easily pipettable liquid. An alternative method of processing is to collect saliva in plastic cups, freeze and thaw it, afther which the saliva samples are sonificated to

obtain a though not entirely clear, yet easily pipettable liquid. In this case the samples were stored in glass tubes.

Reason for this investigation was the fact, that in the first method of processing sometimes inexplicable losses of steroid hormone, particularly progesterone, were found. To investigate the effect of pretreatment several dozens of saliva samples were collected from men, women and children. Each sample was divided into 2 portions, one portion of which was treated with the centrifugation method and the other with the sonification method. With regard to the measured steroids the effect of both methods was compared for cortisol, cortisone, progesterone, 17-hydroxy-progesterone, testosterone, androstenedione and oestradiol.

Chapter 5: Differences between concentrations of salivary cortisol and cortisone and of free cortisol and cortisone in plasma during pregnancy and postpartum.

In Chapter 5 the results of an investigation are described in which in the same study group and with the same methods as in Chapter 3 cortisol and cortisone were measured in plasma (total and free) and in saliva, both in the morning and in the evening. This investigation was intended to demonstrate the relation between corticosteroids in saliva and free in plasma during a period (pregnancy) in which in blood the concentrations of both CBG and total cortisol increase considerably. The samples collected at 6 weeks postpartum served as controls (normal values). Additionally, it was attempted to get insight in the activity of the enzyme 11ß-HSD in the salivary gland and a possible influence of pregnancy on this activity. This was investigated by means of the ratio cortisol : cortisone in plasma (free fraction) as well as in saliva.

Chapter 6: The effect of oral contraceptive use and pregnancy on the daily rhythm of cortisol and cortisone in saliva.

Chapter 6 describes the investigation concerning the effect of use of oral contraceptives (OCs) and of pregnancy in comparison with the normal situation on the daily rhythm of the corticosteroids cortisol and cortisone. OC usage and pregnancy lead to an increase in the concentration of cortisol in plasma to a level comparable to that in Cushing's syndrome. However, in contrast to Cushing's syndrome where the daily rhythm of cortisol usually no longer exists, this rhythm pertains in pregnancy and OC usage.
It is known that the peak of cortisol appears in early morning. In most previous studies for the measurement of plasma free and salivary cortisol, samples were collected between 8 and 10 h, a period of time with marked changes in concentrations of cortisol. Contradicting results may therefore partly be explained by the influence of the daily rhythm.
In this study an investigation was performed to compare the daily rhythm of cortisol in OC using and pregnant women in comparison with a group of normal women. However, because frequent sampling of blood is stressing and because cortisol in saliva is considered to

give a good reflection of plasma free cortisol, the present investigation was conducted on the basis of the measurement of cortisol in saliva. Three groups of women were involved in the study: 10 pregnant women; 11 women using low-estrogen OCs; and 12 normal women. Saliva samples were collected at intervals between 5.00 h until 24.00 h. It was known that the concentration of cortisol in saliva amounts to about 50 % of that free in plasma as a consequence of the conversion into cortisone by the enzyme 11ß-HSD in the salivary gland. Thus cortisone in saliva is partly derived from plasma free cortisone, partly from plasma free cortisol. For this reason cortisone was measured in all samples too.
From a limited number of women it was possible to collect blood samples spread over the day. In these cases plasma total and free cortisol and cortisone were also determined.

Chapter 7: The relation between cortisol and cortisone in saliva and in plasma in various hormonal conditions.

In Chapter 7 the results are described of an investigation regarding the relation between plasma free cortisol and cortisone and salivary cortisol and cortisone and a possible influence of hormonal status on this relation.
The results given in Chapter 5 indicated that the ratio of plasma free to saliva cortisol and cortisone and of the ratio cortisol to cortisone changed in the course of pregnancy. This indicated an influence of factors related to pregnancy on the passage of the corticosteroids from plasma to saliva and on the conversion of cortisol into cortison. To get further insight in the influence of hormonal factors the study was extended to women using low-estrogen oral contraceptives and to a control group. Cortisol and cortisone were measured in plasma (total and free) and saliva in five groups of women: 1) normal, non-pregnant non-OC-using women; 2) pregnant women at the end of the third trimester; 3) women at 6 weeks postpartum; 4) women using Stediril; 5) women using Marvelon.
Besides cortisol and cortisone also CBG was measured in the plasma samples. Values and ratios for cortisol and cortisone were compared for all the study groups.

Chapter 8: General discussion.

In Chapter 8 the results of the overall study were summarized in short. The principal conclusion was that in some situations the concentration of cortisol + cortisone in saliva is higher than would be expected on the basis of the concentration of cortisol + cortisone free in plasma. This finding indicates, that not only the free fraction is transported by diffusion through the salivary gland, but probably also an active proces via specifically binding proteins plays a role herein.
The various possibilities of specifically binding proteins at both the plasma side and at the saliva side of salivary gland cells are discussed, as well as their functioning.

CHAPTER 10

Samenvatting

SAMENVATTING

Hoofdstuk 1: Inleiding

In Hoofdstuk 1 wordt uitgelegd in welke vormen cortisol in plasma voorkomt, namelijk sterk gebonden aan CBG (corticosteroidbindend globuline), matig sterk gebonden aan albumine en als ongebonden, vrij hormoon. Algemeen wordt de vrije vorm gezien als de biologisch actieve component en een verhoging of verlaging van de concentratie vrij cortisol in plasma gaat gepaard met ernstige klinisch aantoonbare afwijkingen. Een verandering in deze concentratie is echter als gevolg van de bindende eiwitten en de werking van een negatief feed-back systeem niet direct af te leiden uit een totale plasmaconcentratie van cortisol. Derhalve is er veel aandacht besteed aan enerzijds de relatie totaal versus vrij plasmacortisol, anderzijds aan de meting van de concentratie vrij cortisol in plasma. Deze laatste heeft namelijk eerder geleid tot tegenstrijdige resultaten, die voor een deel te wijten waren aan de toegepaste analysemethoden.

De aantoning, dat cortisol ook gemeten kan worden in speeksel en dat de speekselwaarden een goede reflectie vormen van vrij plasmacortisol, heeft geleid tot een hele serie onderzoeken. De eindconclusie was, kort samengevat, dat cortisol vanuit de vrije fractie in plasma door simpele diffusie in speeksel terecht komt en daardoor een goede parameter is voor de biologische activiteit ervan.

Het in deze thesis beschreven onderzoek was gericht op de relatie tussen vrij cortisol in plasma en speekselcortisol in condities, dat de totale concentratie van cortisol in plasma duidelijk verhoogd is ten opzichte van normaal, terwijl dit niet gepaard gaat met symptomen die duiden op een verhoging van vrij plasmacortisol. Als inleiding is een uitgebreide literatuurstudie verricht over dit onderwerp en de resultaten zijn samengevat in Tabel 1 en 2.
Ter aanvulling zijn literatuurgegevens betreffende vrij plasmaprogesteron en speekselprogesteron, alsmede gegevens betreffende het voorkomen van en de activiteit van het enzym 11ß-HSD (11-beta-hydroxysteroid dehydrogenase) weergegeven in respectievelijk Tabel 3, 4 en 5. Progesteron werd in de studie betrokken, omdat dit hormoon invloed kan hebben op de binding van cortisol aan plasmaeiwitten en derhalve, ten gevolge van verdringing, op de concentratie van plasma vrij cortisol. 11ß-HSD werd in de literatuurstudie betrokken, omdat dit enzym aanwezig is in de speekselklier, hier cortisol omzet in cortison en als gevolg invloed heeft op de concentratie van speekselcortisol.

Hoofdstuk 2: Het effect van orale contraceptiva op plasma vrij en speeksel cortisol.

In Hoofdstuk 2 worden de resultaten beschreven van een onderzoek naar de effecten van orale contraceptiva (OC's) op de concentraties van plasma-CBG, plasma totaal en vrij cortisol, plasma totaal en vrij cortison, en speekselcortisol en -cortison.
Het is bekend, dat als gevolg van de estrogene component in OC's de concentratie CBG in plasma stijgt. Dit resulteert in een gelijktijdige toename in totaal plasmacortisol, terwijl er in situaties van OC-gebruik geen symptomen van pathologisch verhoogd cortisol worden waargenomen. De verwachting, dat in zo'n geval vrij cortisol in een normaal bereik zal liggen, kon in eerdere studies niet eenduidig worden aangetoond.
In het hier beschreven onderzoek werden de bovengenoemde parameters gemeten met zeer nauwkeurige methoden om na te gaan of vrij cortisol in plasma van OC-gebruikende vrouwen wel of niet verhoogd was ten opzichte van normale waarden (controlegroep). Daarnaast werd bekeken of in plasma gevonden waarden voor vrij cortisol vergelijkbaar waren met die in speeksel. Bovendien werd, gezien de bekende activiteit van 11ß-HSD in de speekselklier, ook cortison gemeten in dezelfde plasma- en speekselmonsters.

Hoofdstuk 3: Speeksel progesteron reflecteert uitstekend vrij en totaal progesteron in plasma tijdens de zwangerschap.

In Hoofdstuk 3 is het onderzoek beschreven naar de relatie tussen plasma totaal en vrij progesteron en speekselprogesteron tijdens de zwangerschap. Progesteron is een hormoon, dat aanzienlijk in concentratie stijgt in de loop van de zwangerschap. Dit steroidhormoon vertoont veel gelijkenis met cortisol wat betreft binding aan plasmaeiwitten en overgang van plasma naar speeksel. Dit was aanleiding om na te gaan in hoeverre speekselwaarden voor progesteron overeenkwamen met de vrije plasmawaarden.
Voor het onderzoek werden plasma- en speekselmonsters verzameld van zwangere vrouwen op 4 tijdstippen tijdens de zwangerschap en ter controle 6 weken post partum. Om de bovengenoemde parameters te meten werden analysemethoden aangepast, verbeterd en ontwikkeld om tot nauwkeurige resultaten te komen.

Hoofdstuk 4: Het effect voor vóórbehandeling van speeksel op steroidhormoonconcentraties.

In Hoofdstuk 4 worden de resultaten gegeven van een onderzoek naar het effect van de voorbewerking van speekselmonsters op de uiteindelijke resultaten van steroidanalysen. Een standaardmethode voor het verzamelen en opwerken van speeksel vóór een analyse is het opvangen van speeksel in plastic potjes, het invriezen en ontdooien van de monsters om ze minder visceus te maken, en daarna het centrifugeren van de monsters in plastic buizen om vaste deeltjes te verwijderen. Bovenstaande vloeistof wordt dan voor de meting gebruikt en bestaat uit een heldere, makkelijk pipetteerbare vloeistof. Een alternatieve methode van

opwerken is het opvangen van speeksel in plastic potjes, invriezen en ontdooien, terwijl daarna de speekselmonsters worden gesonificeerd om een weliswaar niet geheel heldere, maar wel makkelijk pipetteerbare vloeistof te verkrijgen. Het bewaren van de monsters gebeurde in dit geval in glazen buizen.
Aanleiding tot dit onderzoek was het feit, dat met de eerste opwerkingsmethode soms onverklaarbare verliezen van steroidhormoon, met name progesteron, optraden. Om het effect van de voorbewerking te onderzoeken werden vele tientallen speekselmonsters verzameld van mannen, vrouwen en kinderen van zeer uiteenlopende leeftijd. Elk monster werd verdeeld in 2 porties, waarvan de ene portie werd behandeld met de centrifugatiemethode en de andere met de sonificatiemethode. Wat betreft de gemeten steroiden werd het effect van beide methoden vergeleken voor cortisol, cortison, progesteron, 17-hydroxy-progesteron, testosteron, androsteendion en oestradiol.

Hoofdstuk 5: Verschillen tussen concentraties van speeksel cortisol en cortison en van vrij cortisol en cortison in plasma tijdens de zwangerschap en post partum.

In Hoofdstuk 5 worden de resultaten beschreven van een onderzoek, waarbij in dezelfde onderzoeksgroep en met dezelfde werkwijzen als beschreven in Hoofdstuk 3 cortisol en cortison werden gemeten in plasma (totaal en vrij) en in speeksel, zowel 's morgens als 's middags. Met dit onderzoek werd beoogd de relatie aan te tonen tussen de corticosteroiden in speeksel en vrij in plasma tijdens een periode (zwangerschap), dat in het bloed de concentraties van zowel CBG als totaal cortisol enorm toenemen. De monsters die 6 weken post partum verzameld werden, dienden ter controle (normaalwaarden). Daarnaast werd getracht inzicht te krijgen in de activiteit van het enzym 11ß-HSD in de speekselklier en een eventuele invloed van de zwangerschap op deze activiteit. Dit werd onderzocht aan de hand van de verhouding cortisol : cortison zowel in plasma (de vrije fractie) als in speeksel.

Hoofdstuk 6: Het effect van het gebruik van orale contraceptiva en zwangerschap op het dagritme van cortisol en cortison.

In Hoofdstuk 6 wordt het onderzoek beschreven naar het effect van het gebruik van orale contraceptiva (OC's) en van zwangerschap in vergelijking met een groep normale vrouwen op het dagritme van de cortisosteroiden cortisol en cortison. OC-gebruik en zwangerschap leiden tot een toename in de concentratie van cortisol in plasma tot een niveau, dat vergelijkbaar is met dat in Cushing syndroom. In tegenstelling tot Cushing syndroom, waarbij het dagritme van cortisol niet langer bestaat, blijft echter dit ritme behouden tijdens zwangerschap en OC-gebruik.
Het is bekend, dat de piek van cortisol optreedt vroeg in de morgen. In de meeste eerdere onderzoeken voor de meting van plasma vrij en speekselcortisol werden monsters verzameld tussen 8.00 en 10.00 uur, een tijd van grote veranderingen in concentraties van corti-

sol. Tegenstrijdige resultaten kunnen daarom gedeeltelijk verklaard worden door de invloed van het dagritme.
In deze studie werd een onderzoek uitgevoerd om het dagritme van cortisol te vergelijking in OC-gebruikende en zwangere vrouwen in vergelijking met een groep normale vrouwen. Omdat echter frequent afnemen van bloed vervelend is en omdat cortisol in speeksel gezien wordt als een goede afspiegeling van plasma vrij cortisol, werd dit onderzoek gedaam op basis van de meting van cortisol in speeksel. Er werden drie groepen vrouwen betrokken in de studie: 10 zwangere vrouwen, 11 vrouwen die laaggedoseerde OC's gebruikten; en 12 normale vrouwen. Speekselmonsters werden verzameld tussen 5.00 en 24.00 uur. Het was bekend, dat de concentratie cortisol in speeksel ongeveer 50 % bedraagt van die vrij in plasma als gevolg van de omzetting in cortison door het enzym 11ß-HSD in de speekselklier. Cortison in speeksel is dus gedeeltelijk afkomstig van plasma vrij cortison en gedeeltelijk van plasma vrij cortisol. Daarom werd ook cortison gemeten in alle monsters.
Het was mogelijk om van een beperkt aantal vrouwen ook bloed over de dag te verzamelen. In deze gevallen werd ook plasma totaal en vrij cortisol en cortison bepaald.

Hoofdstuk 7: De relatie tussen cortisol en cortison in speeksel en in plasma in diverse hormonale condities.

In Hoofdstuk 7 worden de resultaten beschreven van een onderzoek naar de relatie tussen cortisol en cortison vrij in plasma en in speeksel en de eventuele invloed van hormonale status op deze relatie.
De resultaten, die gegeven worden in Hoofdstuk 5 duiden erop, dat de verhouding plasma vrij tot speeksel cortisol en cortison en de verhouding cortisol tot cortison veranderde in de loop van de zwangerschap. Dit duidde op een invloed van hormonale factoren, die gepaard gaan met zwangerschap, op de overgang van de corticosteroiden van plasma naar speeksel en op de omzetting van cortisol in cortison. Om meer inzicht te krijgen in de invloed van hormonale factoren werd het onderzoek uitgebreid met vrouwen, die laaggedoseerde orale contraceptiva gebruiken en met een controle groep. Cortisol en cortison werden gemeten in plasma (totaal en vrij) en in speeksel bij vijf groepen vrouwen: 1) normale, niet-zwangere, niet-OC-gebruikende vrouwen; 2) zwangere vrouwen aan het einde van het derde trimester; 3) vrouwen 6 weken post partum; 4) vrouwen die Steriril gebruiken; 5) vrouwen die Marvelon gebruiken.
Naast cortisol werd ook CBG gemeten in de plasmamonsters. De waarden en ratio's voor cortisol en cortison werden voor alle groepen vergeleken.

Hoofdstuk 8: Algemene discussie.

In Hoofdstuk 8 worden in het kort de resultaten van het totale onderzoek samengevat. De voornaamste conclusie was, dat in sommige gevallen de concentratie cortisol + cortison in speeksel hoger was dan verwacht kon worden op basis van de concentratie cortisol + cortison vrij in plasma. Deze bevinding duidt erop, dat niet alleen de vrije fractie door middel

van diffusie getransporteerd worden door de speekselklier, maar dat waarschijnlijk ook een actief proces via specifiek bindende eiwitten hierbij een rol speelt.
De diverse mogelijkheden van specifiek bindende eiwitten aan zowel de plasmakant als aan de speekselkant van speekselkliercellen, alsmede de werking ervan worden uitvoerig besproken.

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CURRICULUM VITAE

De schrijfster dezes werd geboren op 28 september 1952 in Heerlen. Na het doorlopen van de lagere meisjesschool in Nieuw-Lotbroek werd in 1971 op het St. Janscollege in Hoensbroek het diploma gymnasium-ß behaald. Daarna volgde een studie biologie aan de Katholieke Universiteit van Nijmegen, welke afgesloten werd met het doctoraal in 1977. Na 1 jaar op de afdeling Moleculaire Biologie van de KUN in het kader van een TAP-regeling kreeg zij de mogelijkheid door middel van herhaalde tijdelijke aanstellingen te werken op de afdeling Endocrinologie van de Faculteit Geneeskunde/St. Radboud Ziekenhuis. Van 1987 tot 1989 volgde een aanstelling als wetenschappelijk medewerkster op de afdeling Gynecologie. In 1990 werd de overstap gewaagd naar een eigen bedrijf, waar zij zich tot op heden bezig houdt met onderzoek op het gebied van milieuverontreinigende stoffen en met name de immunochemische analyse hiervan.