Salivary Cortisol in Psychobiological Research: An Overview

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Key Words. Saliva • Cortisol • Hormones • Hypothalamus-pituitary-adrenal axis • Steroid hormones • Stress • Immunoassay • Dexamethasone suppression test • Corticotropin-releasing hormone challenge

Abstract. The measurement of cortisol in saliva provides the basic scientist as well as the clinician with a reliable tool for investigations of hypothalamus-pituitary-adrenal axis activity. Since saliva samples can be obtained stress-free and independent from medically trained personnel this method may be well suited for use in psychobiological studies. This overview intends to give a comprehensive introduction to the method of salivary cortisol assessment and to briefly discuss its application in different scientific disciplines.

Introduction

Throughout the last decade the use of saliva as an alternative specimen for the determination of hormones has gained increasing attention. Researchers and clinicians in endocrinology, psychobiology, behavioral medicine, and psychiatry successfully applied the measurement of steroids in saliva and pointed out the potential advantages of this method over blood analyses. Over 400 studies are now available which suggest that the measurement of cortisol, estriol, progesterone, testosterone, and cortisone in saliva is a reliable reflection of the plasma values of the respective hormone, being partly reviewed elsewhere [Riad-Fahmy et al., 1982, 1987; Vining and McGinley, 1986, 1987].

Although it is well documented that other steroid hormones are also affected by stress, cortisol is still considered a major indicator of altered physiological states in response to stressful stimulation. In the past, most of the studies conducted with humans on this issue faced numerous problems associated with venipuncture for blood sampling. Besides the reactivity of the method, ethical and practical constraints (studies in infants and children; multiple sampling over longer time periods) as well as economical factors have hampered research on the psychoendocrinology of the human cortisol system. However, recent developments in biochemical assays and progress in methodology now offer the tools to circumvent these difficulties by employing reliable and convenient measurement of cortisol in saliva. As outlined below in greater detail, salivary cortisol determinations bear characteristics which could make this technique the method of choice in psychoendocrinological studies.

Following the pioneering studies of Ira Shannon and Fred Katz on the measurement of corticosteroids in saliva [Shannon et al., 1959a, b, 1966; Katz and Shannon, 1964] this monitoring procedure gained only little acceptance due to several methodological problems [Riad-Fahmy et al., 1982]. The development of sensitive radioimmunoassay (RIA) techniques in the late 70s rekindled interest in monitoring cortisol in saliva, and the

1 This paper is dedicated to Professor emerita Dr. Lilly Kemmler, who supported the work from its very beginning.
growing attention being drawn on salivary cortisol measurement may be indicated by the number of papers published on this issue throughout the last decade (fig. 1). However, there is still a great deal of uncertainty among researchers and clinicians concerning the validity of the method. It appears that most reservations may be dispelled by a thorough review of the literature. Furthermore, the present overview may help to encourage further research on the relationships between hypothalamic-pituitary-adrenal (HPA) activity and behavior employing salivary cortisol measurements.

**Basic Issues**

Cortisol is the main glucocorticoid hormone in humans being produced in the adrenal cortex. It is released both spontaneously [Van Cauter, 1987] and in response to various biochemical agents and psychosocial stimuli. Following secretion approximately 90% of the endogenous hormone is bound to the blood-borne carriers corticosteroid-binding globulin (CBG or transcortin) and albumin, while only 5–10% of the hormone circulates unbound ('free'). CBG expresses high-affinity receptors, binding 70–85% of the total cortisol, while albumin only binds 10–15% by low-affinity binding structures. Furthermore, Hiramatsu and Nisula [1988] have recently shown that cortisol is also bound to erythrocyte membranes to a considerable extent. The authors estimated that the proportion of cortisol carried by red blood cells equals the free plasma hormone fraction. It is only the latter fraction which is believed to be the biologically active one [Robbins and Rall, 1957; Ekins, 1990]. Thus, only 5–10% of the total cortisol acts upon the target tissues, leading to a broad spectrum of physiological effects, since virtually all body cells are affected by cortisol [for detailed reviews see Munck et al., 1984; Bondy, 1985].

**Relationship between Plasma Unbound Cortisol and Salivary Cortisol**

One major criticism against the use of saliva instead of plasma for cortisol determinations is that values in saliva may not accurately represent the concentrations found in blood. Like any other steroid hormone, cortisol is highly lipid-soluble and together with its small size (molecular weight 362), this property enables the molecule to rapidly diffuse through the lipid-rich cell membranes via passive intracellular diffusion. Once carried to the acinar cells of the secretory endpiece of the salivary glands by the blood stream, cortisol and other unconjugated steroids can easily pass through these cells into saliva. Therefore, it is not surprising that saliva flow rate has no impact on salivary cortisol levels. Several research groups have carefully looked at hormone concentrations in saliva at different flow rates. Neither maximal stimulation of saliva flow by administration of citric acid to the tongue [Walker et al., 1978, 1984; Ferguson et al., 1980; Al-Ansari et al., 1982; Vining et al., 1983a; Vining and McGinley, 1984; Ben-Aryeh et al., 1985; Dirks et al., 1988; Kahn et al., 1988] nor minimal secretion of saliva following medication with anticholinergic drugs ('dry mouth') [Cook et al., 1986a] influenced the concentration of cortisol in saliva significantly. This is especially important for the clinician working with anxiety patients or phobics, since changes in composition, viscosity, and flow rate of saliva can be observed in these patients due to higher sympathetic arousal. The linear correlations between cortisol levels in the two body fluids are high, ranging from $r = 0.54$ to $r = 0.97$, with most investigators reporting on correlation coefficients of $r \geq 0.90$, so more than 80% of the total variance are usually determined by this correlation [Ferguson et al., 1980; Hiramatsu, 1981; Vining et al., 1983a; Atkinson et al., 1984; Walker et al., 1984; Allolio et al., 1985; Burke et al., 1985; Fell et al., 1985; Hanada et al., 1985; Luthold et al., 1985; Cook et al., 1986a, b; Francis et al., 1987; Kahn et al., 1988]. Typically, lower correlation coefficients are reported if total plasma cortisol (and not plasma free) values are compared to salivary levels. One reason for this discrepancy might be that under various circumstances.
Conditions the amount of CBG and/or albumin is altered with concurrent elevated levels of total cortisol but no apparent alteration of the amount of the free hormone fraction (see below). Considering that several percent of the remaining variance are determined by the unreliability of the analytical technique these data strongly suggest that salivary cortisol is a good reflection of the plasma unbound moiety.

As for the time lag between an increase in plasma cortisol compared to saliva, Walker et al. [1984] observed a rapid transfer of cortisol from blood. After injection of 5 mg cortisol to 2 normal, dexamethasone-suppressed males they found a significant increase in salivary cortisol levels within the first minute following steroid administration. Peak values in saliva were observed 1–2 min following maximal concentrations in plasma.

Two different results on the half-life of salivary cortisol have been reported in the literature. While Hiramatsu [1981] reported a half-life of 58 min in saliva and 72 min in plasma (unbound fraction), another set of data exists suggesting a half-life of 106–113 min for the disappearance of cortisol in saliva with no difference compared to plasma free cortisol [Evans et al., 1984, 1985; Peters et al., 1984]. The contrasting results may, in part, be affected by methodology. Hiramatsu [1981] injected his subjects with cortisol intravenously while Evans et al. [1984] and Peters et al. [1984] calculated the disappearance following continuous dexamethasone infusion.

Cortisol Secretion and Circadian Rhythm

Like the majority of hormones, cortisol is not secreted in a continuous flow from the adrenal cortex but in a pulsatile fashion resulting in several secretory episodes throughout the day. In undisturbed subjects there are typically about 15 distinct cortisol pulses detectable within 24 h with temporal adjustment to a given environment by a Zeitgeber. In healthy subjects the adrenal cortex is stimulated and inhibited in an on-off fashion, i.e. secretory episodes are followed by periods of glandular inactivity [Van Cauter, 1987].

This pattern of normal cortisol secretion is regulated by two negative, open-loop feedback systems, a fast rate-sensitive and a delayed proportional control system [Fehm et al., 1979; Brandenberger et al., 1984]. In concert with a Zeitgeber (which is believed to be located in the hypothalamus) the feedback systems determine the circadian hormone profile. Pulse amplitude is highest in early morning hours and small in the evening, resulting in the well-documented circadian rhythmicity of cortisol output. A clear-cut circadian change of salivary cortisol levels with the highest concentrations in early morning hours and the lowest values around midnight is found in normal subjects. This pattern closely parallels the respective changes in plasma. Walker et al. [1984] studied the intraindividual stability of the rhythm by a 2-hour sampling interval during the waking hours in 5 healthy volunteers for 5 consecutive days. They reported a marked similarity of cortisol profiles between days [Walker et al., 1984] suggesting a rather high stability of this pattern. However, in two studies with 48 students and 54 young mothers, respectively, we found considerable intraindividual variability in salivary cortisol across days [Kirschbaum et al., in press]. Only the early morning measures showed a rather high stability over 3 days while afternoon and evening values appeared to be strongly influenced by external stimulation.

The time of first appearance of the circadian rhythm in the infant has been discussed controversially. While Franks [1967] suspected that the pattern is first present between 1 and 3 years of age, Price et al. [1983] observed the appearance of a normal cortisol circadian rhythm by the 3rd month, which coincided with or preceded the development of a regular night sleep in 6 out of 8 term infants investigated. In their study Price et al. [1983] took advantage of one of the predominant features of salivary cortisol measures: mothers were instructed to obtain saliva samples from their infants by simply aspirating saliva with a disposable mucus extractor at home. Sampling was performed stress-free in the natural environment of the infants, thus leading to results of enhanced validity.

Normal Values

Although the hormone concentration parallels of the free fraction in plasma, absolute salivary cortisol levels are up to 50% lower compared to the active molecule in blood [Vining et al., 1983a; Brooks and Brooks, 1984; Meulenbergen et al., 1987]. This is caused by 11β-hydroxysteroid dehydrogenase, an enzyme converting cortisol to cortisone, which is present in large amounts in saliva.

A comparison of absolute hormone concentrations between laboratories is sometimes difficult if different assay systems for hormone measurement are used. Due to several factors, e.g. significant cross-reactivity of the antiserum may lead to higher/lower absolute hormone
levels compared to results from other laboratories. Obviously, this problem occurs with saliva samples as well as with blood samples [Seth, 1987]. While for plasma/serum cortisol there are quality control samples with known cortisol concentration commercially available which allow for calibration of the assay employed, there is no standardized reference material available for salivary cortisol. Each laboratory has to carefully evaluate its analytical material as for the normal values found in a given population. However, this procedure hinders a direct comparison of results obtained in different research groups. Investigating the absolute concentrations obtained with a given analytical reagent kit we measured 36 saliva samples with four RIA systems to compare the absolute values obtained. Though there were excellent correlations among the concentrations measured, absolute cortisol levels differed by up to 220% between assays in identical samples (fig. 2) [Kirschbaum et al., 1989b]. Hence, special care is called for if absolute salivary cortisol levels obtained with different assays are to be compared. The development and distribution of reference material would be most helpful for laboratories and investigators for optimum calibration of the assay performed.

Table 1 summarizes early morning salivary cortisol concentrations measured in healthy subjects and pathological values from patient populations with disorders associated with alteration of HPA activity. The wide range of mean salivary cortisol levels in unstimulated early morning samples with values from 8.3 [McVie et al., 1979] to 27.3 nmol/l [Umeda et al., 1981] reflects differences in analytical technique rather than a genuine variation of glandular activity. Values from patients with Cushing’s disease are well separated from healthy controls showing 2 to 3-fold elevation in salivary cortisol levels. Interestingly, recent data from Guéchot et al. [1987] suggest that patients suffering from major depression may be separated from secondary depressive patients by measuring cortisol in saliva at 11 p.m. While the latter show normal values of 2.2 nmol/l, primary depressive patients had mean salivary cortisol concentration of 5.0 nmol/l, indicating HPA hyperactivity in some patients. Replication studies and cross-validation are necessary to support the authors’ view of the 11 p.m. measure as a ‘simple laboratory test of neuroendocrine disturbance in depression’.

While no differences in salivary cortisol levels between sexes were found by several research groups [Guéchot et al., 1981, 1982; Riad-Fahmy et al., 1982; Bustamante and Crabbe, 1984; Kugler and Kalveram, 1989], Laudat et al. [1988] reported on a two-fold higher concentration in unstimulated early morning samples of 23 healthy males compared to 35 women. Possibly the high interassay coefficient of variance (14%) of the assay used by this group has caused this discrepant result. In a recent large-scale study at the University of Trier, FRG, with 720 healthy adults who delivered morning, after-

Fig. 2. Comparison of three different coated tube RIAs with the adapted Magic Cor RIA. [Figure taken from Kirschbaum et al., 1989; reprinted with permission.]
<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Subjects</th>
<th>Age years</th>
<th>Cortisol in saliva 8–9 a.m. nmol/l</th>
<th>Assay used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al-Ansari et al. [1982]</td>
<td>10 healthy adults</td>
<td>?</td>
<td>12.5</td>
<td>modified 'Corning Immunophase Cortisol RIA'</td>
</tr>
<tr>
<td>Anseau et al. [1984]</td>
<td>15 major depressives 11 control patients</td>
<td>18–66 27–63</td>
<td>13.9 11.4</td>
<td>in-house antiserum + 'Famos' tracer</td>
</tr>
<tr>
<td>Bustamante and Crabbe [1984]</td>
<td>12 healthy adults</td>
<td>22–32</td>
<td>14.8</td>
<td>modified assay from Radioassay Systems Labs</td>
</tr>
<tr>
<td>Cook et al. [1986b]</td>
<td>8 marathon runners</td>
<td>27–49</td>
<td>23.5</td>
<td>in-house RIA</td>
</tr>
<tr>
<td>Guéchot et al. [1981]</td>
<td>71 healthy adults 4 Cushing patients 23 healthy females, contraceptive pill 31 pregnant women, 3rd trimester</td>
<td>?</td>
<td>13.7 15.7 11.4 16.0</td>
<td>(method not given)</td>
</tr>
<tr>
<td>Guéchot et al. [1982]</td>
<td>19 healthy males 45 untreated females 25 healthy females, contraceptive pill 36 pregnant women, 3rd trimester</td>
<td>20–45 18–40 18–40 18–40</td>
<td>11.3 14.0 13.4 14.2</td>
<td>in-house RIA</td>
</tr>
<tr>
<td>Hiramatsu [1981]</td>
<td>8 healthy adults</td>
<td>24–33</td>
<td>9.7</td>
<td>modified 'SPAC Cortisol'</td>
</tr>
<tr>
<td>Kahn et al. [1984]</td>
<td>9 healthy adults 3 Cushing patients</td>
<td>22–43</td>
<td>14.7</td>
<td>in-house RIA</td>
</tr>
<tr>
<td>Luthold et al. [1985]</td>
<td>8 healthy adults</td>
<td>20–43</td>
<td>14.0</td>
<td>in-house RIA</td>
</tr>
<tr>
<td>McVie et al. [1979]</td>
<td>10 healthy adults</td>
<td>?</td>
<td>8.3</td>
<td>in-house RIA</td>
</tr>
<tr>
<td>Price et al. [1983]</td>
<td>8 healthy children</td>
<td>0.5</td>
<td>12.7</td>
<td>in-house RIA</td>
</tr>
<tr>
<td>Peters et al. [1984]</td>
<td>16 healthy adults</td>
<td>?</td>
<td>12.0</td>
<td>in-house RIA</td>
</tr>
<tr>
<td>Umeda et al. [1981]</td>
<td>10 healthy males</td>
<td>24–33</td>
<td>27.3</td>
<td>in-house RIA</td>
</tr>
<tr>
<td>Vining et al. [1983b]</td>
<td>14 healthy adults</td>
<td>24–32</td>
<td>15.2</td>
<td>in-house RIA</td>
</tr>
</tbody>
</table>

* Age was not differentiated between groups.
* Values interpolated from figure.

noon, and evening saliva samples we found evidence for systematically altered unstimulated cortisol levels between sexes or different age-groups [Brandtstädter et al., unpublished data]. While in men the mean salivary cortisol levels were not altered at different ages, women showed a steady decline of cortisol concentration with increasing age. Treating the group as a whole, however, the values obtained are comparable to hormone concentrations in children (>0.5 years). In agreement with other research groups [Riad-Fahmy et al., 1982, who verified their RIA data by comparing these results with those obtained by gas chromatography-mass spectrometry analysis] (see also table 1) we found the following salivary cortisol concentration for the total group of adults (means ± 1 SD):

<table>
<thead>
<tr>
<th>Time</th>
<th>Concentration (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7–9 a.m.</td>
<td>14.32 ± 9.1 (n = 662)</td>
</tr>
<tr>
<td>3–5 p.m.</td>
<td>4.50 ± 3.5 (n = 708)</td>
</tr>
<tr>
<td>8–10 p.m.</td>
<td>1.96 ± 1.7 (n = 698)</td>
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</table>
Pregnancy, Oral Contraceptives, and Salivary Cortisol

The synthesis of CBG in the liver is enhanced under several physiological conditions, for example in pregnancy or during the intake of estrogen-containing contraceptive medication. Since CBG-bound cortisol cannot be metabolized in the liver, circulating levels of cortisol in plasma are markedly elevated under those conditions [Vining et al., 1983a; Landon et al., 1984]. Although the total steroid concentration can reach cushingoid levels the women do not show clinical symptoms of hypercortisolism because a feedback mechanism keeps the biologically active free cortisol fraction well within the normal range.

In saliva only 0.1% of total plasma CBG levels are usually found in unstimulated samples [Hammond and Langley, 1986] and apparently no binding globulin is detectable after stimulation of saliva flow rate above 300 µl/gland/min [Chu and Ekins, 1988]. In normal subjects CBG in saliva increases cortisol levels up to 10–15% maximally, but Chu and Ekins [1988] suspected that in pregnancy and in estrogen-taking women these circumstances might be more important, leading to erroneous results.

However, data on salivary cortisol levels in pregnant women are contradictory. While several investigators reported on unchanged steroid levels in this population [Guéchot et al., 1981, 1982; Landon et al., 1984; Peters et al., 1984] another set of data supports the view of elevated cortisol concentrations at least in the third trimester [Katz and Shannon, 1964; Stahl and Dörner, 1982; Vining et al., 1983b; Bustamante and Crabbe, 1984; Laudat et al., 1988]. The latter findings may be explained by the large increase in progesterone levels which competes with cortisol for binding sites on CBG and at the intracellular level in target cells [Ballard, 1979; Raynaud et al., 1980]. On the other hand unchanged salivary cortisol levels in pregnancy would again underscore the validity of the free hormone concept [Robbins and Rall, 1957; Ekins, 1990]. The present results do not allow an ultimate judgement whether or not cortisol levels are enhanced in unstimulated saliva from pregnant women because those studies which failed to reveal the respective difference in hormone concentration had too small population samples to allow the interpretation of the zero hypothesis. Furthermore, due to the more or less large interindividual variations it would be advantageous to have multiple samples from each subject as in the study of Vining et al. [1983b], who obtained 15 samples from each third-trimester woman at hourly intervals from 6 a.m. to 9 p.m. Using this or similar protocols more powerful statistical tests could be applied for data analysis to unmask the potential effect. For example, cortisol levels may be unaltered in afternoon or early evening samples in these women while morning cortisol levels are elevated. Study designs with repeated measurements could give a more valid estimation of the total amount of bioavailable cortisol than single Student’s t tests in his case. Until results from such studies exist one appears to be well-advised to exclude those women from studies where absolute salivary cortisol values are compared.

The impact of estrogen-containing medication on cortisol in saliva has also gained considerable attention. Total plasma cortisol concentration rises following stimulation of hepatic CBG synthesis (see above) by estrogens. A comparable increase in salivary cortisol, however, has not been measured in a number of studies [Vining et al., 1983a; Evans et al., 1984; Alloio et al., 1986]. Recently, Meulenber et al. [1987] published results on salivary cortisol values in 23 women who were on a low-estrogen medication (30 µg ethinyl estradiol + 150 µg desogestrel; 'pill group') for at least 6 months. Compared to 15 drug-free control subjects the pill group showed a twofold increased cortisol concentration on the 18th day of a pill cycle. Meulenber et al. [1987] used sophisticated biochemical techniques for hormone determination, thus the striking differences between their data and former reports may not be ascribed to analytical problems. Surprisingly, in their paper they did not mention that both groups failed to show different hormone values during the first half of the pill/menstrual cycle and that similar values were obtained at other times of day on the 18th day [Meulenber, personal commun.]. To follow up the proposed changes in salivary cortisol levels throughout the course of a pill cycle and the potential impact of day time, we studied 19 women on estrogen contraceptives and compared the absolute cortisol levels in saliva to 16 drug-free controls. Each woman sampled saliva at 9 a.m. every second morning over a period of 30 days and additionally at 12 a.m. and 4 p.m. on day 18 to investigate whether the circadian profile might be altered by the drug. As shown in figure 3 the pill group did not differ significantly in salivary cortisol levels on any of the 15 days where measurements were taken [Kirschbaum and Hellhammer, unpublished data].

Taken together reasonable evidence suggests that estrogen-containing oral contraceptives do not lead to a state of hypercortisolism due to increased plasma CBG.
The independence from binding protein levels seen in these women gives further support to the hypothesis that an increase in cortisol in saliva during pregnancy rather reflects the displacement by competing molecules than a CBG effect.

**Other Substances Influencing Salivary Cortisol Levels**

The analytical technique most frequently employed in assaying cortisol is RIA. Antisera used for RIA cortisol determination can have a considerable cross-reactivity with related structures like prednisolone and metyrapone. Thus, results of saliva samples are deceptive in patients receiving either medication. On the other hand neither anticonvulsant phenytoin-containing drugs [Evans et al., 1985], nor thyroid hormones [Laudat et al., 1988], or tricyclic or quadricyclic antidepressants [Cook et al., 1986a] appear to have a significant impact on cortisol levels in saliva. As for the influence of nicotine, it is generally believed that smoking induces an increase in total plasma cortisol levels [for reviews see Fuxe et al., 1989; Pomerleau and Rocecrans, 1989]. Although Cherek et al. [1982] failed to find any alteration in salivary cortisol levels in 4 healthy smokers, these data do not indicate an absent cortisol response in saliva following nicotine exposure. In line with findings on total plasma cortisol we found a dose-dependent increase in salivary cortisol in approximately 50% of smokers investigated (unpublished data). In this study several subjects showed peak salivary cortisol levels which were 2- to 5-fold elevated over baseline values for more than 30 min after cessation of smoking. Thus it appears that nicotine consumption has to be considered as a potent stimulus for cortisol secretion and as a critical intervening variable.

**Salivary Cortisol and Stress**

It has been known for a long time that the HPA axis can respond sensitively to external stimulation. A variety of agents and treatments (called stressors) are able to override the feedback systems, leading to enhanced frequency and amplitude of cortisol pulses. Multiple approaches have been employed to elucidate the nature of the provoked spontaneous secretory episodes and to relate the hypersecretion of cortisol to cognition, mood, and behavior shown by the individual. However, researchers often encounter ethical and methodological problems when blood sampling is required for cortisol assessment. It is suggested that many of these problems can be circumvented by measuring cortisol in saliva.

One indispensable prerequisite of any investigation of stress effects on the cortisol system is the nonreactivity of the sampling method, i.e., that the sampling procedure does not affect the cortisol values. As shown below, venipuncture can significantly enhance cortisol concentrations in some of the patients investigated, most probably reflecting a psychological stress response. This response can either lead to a state of low reactivity of the whole HPA axis [Follenius and Brandenberger, 1986] or even mask a reaction to subsequent stimuli. Another shortcoming of plasma cortisol in stress research is the inconvenience of sampling. Besides the emotive bias of many individuals against blood drawings, medically trained personnel is always required. While in the laboratory the personnel is often available, studies in a natural environment are costly. In contrast, saliva can easily be obtained by the volunteers or patients themselves. Either in the presence of the investigator or independently under various circumstances samples are provided within less than 1 min and there is no evidence for altered cortisol levels following this procedure.

Besides practical considerations, validity problems may be associated with serum/plasma cortisol measures. Since the determination of free cortisol in serum or
plasma is time-consuming and demands considerable expertise in biochemical analyses, most researchers and clinicians have to be satisfied with total cortisol assessments when merely blood samples were obtained from volunteer subjects or patients. These values may be misleading under several physiological and/or pharmacological conditions. As outlined in the first chapter, cortisol in saliva is unaffected in most of these cases known to systematically increase or reduce the concentration of the total hormone in plasma. Moreover, only the unbound fraction is able to penetrate the membranes of potential target cells on which the hormone exerts its physiological actions. Unless the free cortisol is measured, the assessment of salivary cortisol appears to be the 'better measure of adrenal cortical function than serum cortisol' [Vining et al., 1983a, p. 329]. However, despite obvious advantages of the method rather few papers have been published on salivary cortisol measures under stress so far. Table 2 gives a comprehensive overview of the relevant papers.

**Physical Stress**

Salivary cortisol has been shown to increase in response to different types of physical strain or exercise. Stahli and Dörner [1982] were the first to employ this method to monitor physiological responses to stress situations. They reported increases in salivary cortisol levels after painful medical exploring methods like prostatic biopsy, cystoscopy, and sternal puncture. The magnitude of response was 157, 181, and 203% above baseline val-

<table>
<thead>
<tr>
<th>Authors</th>
<th>Stressed subjects (age)</th>
<th>Control subjects (age)</th>
<th>Stress situation (duration)</th>
<th>Salivary cortisol response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bassett et al. [1987]</td>
<td>29 healthy bank employees (25–36 years)</td>
<td>none; 2nd day taken as a control for stress response</td>
<td>public speaking (15 min)</td>
<td>3 nmol/l (before) and 7 nmol/l (after speaking) higher than on control day</td>
</tr>
<tr>
<td>Ben-Aryeh et al. [1985]</td>
<td>51 women in waiting room (41.6 ± 11 years)</td>
<td>55 women at home (44.1 ± 10 years)</td>
<td>waiting for fine-needle aspiration of breast</td>
<td>5.42 nmol/l (patients) vs. 3.41 nmol/l (controls)</td>
</tr>
<tr>
<td>Cook et al. [1986]</td>
<td>8 healthy men (35.1 ± 8.1)</td>
<td>none; rest days taken as a control for response</td>
<td>marathon run</td>
<td>mean levels before run 6.6 nmol/l higher than on control days; stress peak concentration: 87.9 nmol/l 30 min after completion of the run</td>
</tr>
<tr>
<td>Fell et al. [1985]</td>
<td>8 wethers</td>
<td>none</td>
<td>transport on trailer (30 min)</td>
<td>1.8 nmol/l in race 21.9 nmol/l on trailer after transport</td>
</tr>
<tr>
<td>Fell et al. [1986]</td>
<td>19 calves (7 weeks old)</td>
<td>8 calves (similar age)</td>
<td>surgical castration (n = 9) or castration by application of rubber ring (n = 10)</td>
<td>cortisol values 1 h after castration: 10.2 nmol/l (surgical), 3.2 nmol/l (rubber ring) vs. 1.1 nmol/l (controls); 4 h after treatment all groups had normal concentrations</td>
</tr>
<tr>
<td>Fell and Shutt [1986]</td>
<td>28 calves</td>
<td>9 calves</td>
<td>trucking maneuvers (30 min) and subsequent road transport (2 h)</td>
<td>0.3 nmol/l (baseline) and 3.4 nmol/l (after stress) vs. 0.5 nmol/l (controls)</td>
</tr>
<tr>
<td>Fibiger et al. [1986]</td>
<td>8 male students (18–32 years)</td>
<td>none</td>
<td>3 mental arithmetic tests of graded difficulty (20 min)</td>
<td>no significant alterations of salivary cortisol levels</td>
</tr>
<tr>
<td>Harris et al. [1988]</td>
<td>55 patients in a psychiatric ward</td>
<td>none</td>
<td>venipuncture</td>
<td>no significant alterations of salivary cortisol; means: 3.24 nmol/l (before) vs. 3.30 nmol/l (10 min after venipuncture)</td>
</tr>
<tr>
<td>Hellhammer et al. [1985]</td>
<td>10 male students (24–33 years)</td>
<td>none; samples obtained 1 week after last exam taken as control values</td>
<td>final academic examination</td>
<td>48 nmol/l (mean cortisol levels before exams) vs. 31.5 nmol/l</td>
</tr>
</tbody>
</table>

(Table 2 continued next page)
<table>
<thead>
<tr>
<th>Authors</th>
<th>Stressed subjects (age)</th>
<th>Control subjects (age)</th>
<th>Stress situation (duration)</th>
<th>Salivary cortisol response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hellhammer et al. [1986]</td>
<td>12 healthy adults (21–35 years)</td>
<td>none</td>
<td>2 different films ('Psycho' vs. 'Goldfinger')</td>
<td>significantly higher cortisol levels in response to 'Psycho', reflecting suspenseful anticipation</td>
</tr>
<tr>
<td>Hellhammer et al. [1988]</td>
<td>60 healthy adults (18–24 years) in three groups with different medication on three occasions: (a) 1 mg lorazepam, (b) 0.5 mg alprazolam, (c) placebo</td>
<td>see experimental group (c)</td>
<td>scenes from 'Shining' and 'Halloween' (10 min each)</td>
<td>no overall effect of benzodiazepines on salivary cortisol in stress response observed; only lorazepam appeared to prevent a stress response</td>
</tr>
<tr>
<td>Hubert and Nieschlag [1988]</td>
<td>17 healthy adults (22–34 years)</td>
<td>none</td>
<td>venipuncture</td>
<td>6 responders, 11 nonresponders; in responders salivary cortisol rose from 6.0 nmol/l (baseline) to 12.8 nmol/l (25 min after venipuncture)</td>
</tr>
<tr>
<td>Hubert and de Jong-Meyer [1989]</td>
<td>27 healthy males (18–32 years)</td>
<td>none</td>
<td>suspense film 'Shining' (120 min)</td>
<td>13 responders, 14 nonresponders peak salivary cortisol concentration; 13 nmol/l (20 min after end of film vs. 11 nmol/l (lowest value of 10 consecutive measures)</td>
</tr>
<tr>
<td>Jones et al. [1986]</td>
<td>40 first-year medical students</td>
<td>none; samples on 3 consecutive days prior to examination served as controls</td>
<td>first major examination in medical school</td>
<td>elevated salivary cortisol levels on day of examination; interactions of cortisol response, type A/B, and test performance</td>
</tr>
<tr>
<td>Kakimoto [1985]</td>
<td>6 captains, 6 copilots, and 5 flight engineers</td>
<td>none</td>
<td>multiple takeoffs and landings with cargo airplane</td>
<td>elevated salivary cortisol levels following maneuvers; copilots appeared to show higher responses than captains</td>
</tr>
<tr>
<td>Kirschbaum et al. [1989a]</td>
<td>18 children (9–14 years)</td>
<td>none; response to stress film compared to changes in response to control</td>
<td>scenes from 'Roller-coasters' (10 min)</td>
<td>high-anxiety children showed elevated salivary cortisol levels in response to both films (+27.6 and +38.9%), the low-anxiety children showed decreasing cortisol concentrations (~8.5 and ~20.5%)</td>
</tr>
<tr>
<td>Lehnert et al. [1989]</td>
<td>12 healthy males</td>
<td>none</td>
<td>(a) mental arithmetics, (b) public speaking</td>
<td>(a) unchanged salivary cortisol, (b) elevated salivary cortisol levels in anticipation of task</td>
</tr>
<tr>
<td>O'Connor and Corrigan [1987]</td>
<td>8 male subjects (22.9±2.3 years)</td>
<td>none; cortisol levels in quiet rest condition served as controls</td>
<td>exercise on bicycle ergometer at 75% VO2max (30 min)</td>
<td>20.7 nmol/l (before) vs. 41.4 nmol/l (45 min after start of exercise); no habituation of response upon reexposure to same situation</td>
</tr>
<tr>
<td>Stahl and Dörner [1982]</td>
<td>26 patients in three groups 11 healthy students</td>
<td>15 healthy subjects</td>
<td>painful medical examinations; (a) prostatic biopsy, (b) cystoscopy, (c) sternal puncture, (d) academic examination</td>
<td>mean salivary cortisol levels after stress: (a) 12.1 nmol/l, (b) 16.0 nmol/l, (c) 16.3 nmol/l, (d) 13.8 nmol/l vs. 7.7–8.8 nmol/l (baseline values)</td>
</tr>
<tr>
<td>Tarui and Nakamura [1987]</td>
<td>4 healthy males (22–58 years)</td>
<td>none</td>
<td>acceleration stress at +3 Gs, +4 Gs, and +5 Gs (1 min)</td>
<td>no alterations at +3 Gs; peak cortisol concentration after +5 Gs: 64.9 vs. 22.1 nmol/l (baseline)</td>
</tr>
</tbody>
</table>
ues, respectively. In line with these findings, castration in calves has been found to be associated with elevated salivary cortisol levels, too [Fell et al., 1986].

Using a painless stress model, Tarui and Nakamura [1987] investigated the impact of acceleration on hormone output in 4 healthy nonaircrew volunteers. On 3 different days subjects were exposed to acceleration stress of +3 G, +4 G, and +5 G without a G-suit for 1 min. At the maximal stress of +5 G the authors monitored cortisol levels being threefold elevated in all subjects while minimal or no changes were observed with lower stimulation. This result corroborates earlier findings suggesting a severity-dependent rise of cortisol. In studies employing bicycle ergometer stress volunteers show reliable cortisol responses only if they work at 70% or more of their maximal oxygen uptake (VO₂ max) [Mason et al., 1973]. O’Connor and Corrigan [1987] could show that ergometer exercise at 75% VO₂ max for 30 min results in profound salivary cortisol elevations. Interestingly, this response did not habituate upon repeated exposure. Data from our laboratory further strengthen this hypothesis. In a pilot study on habituation of cortisol responses 20 healthy well-trained men sampled saliva before and after a 5-km race on two occasions 1 week apart. We monitored large increases in salivary cortisol release following the race with mean peak values being 2.5-fold elevated above baseline concentrations (fig. 4). This result was not surprising since Cook et al. [1986b] already reported on respective responses following marathon runs. However, the interesting finding was the similarity of cortisol patterns. The group’s mean baseline concentrations as well as peak and poststress values were almost identical on both days, indicating a rather stable response of the adrenal cortex. Taken together the literature strongly suggests that physical exercise above a threshold of 70% VO₂ max is accompanied by an elevation of salivary cortisol levels in normal individuals. There is no evidence for an attenuation of the response upon reexposure. What is the physiological rationale for this phenomenon? Obviously, intense muscular work is highly energy-consuming, resulting in a reduction of bodily energy reserves. Among other effects in the organism predominant features of cortisol action are gluconeogenesis and enhancement of energy production by different mechanisms [Munck et al., 1984]. Any process which leads to a profound decrease in available energy may thus stimulate synthesis and release of cortisol. Due to these metabolic requirements there is no reason for assuming that cortisol responses will habituate to severe physical stress.

![Salivary cortisol levels of 20 healthy males in response to two 5-km runs (20 min) with a 7-day interval between trials.](image)

**Psychological Stress**

Spontaneous secretory episodes of cortisol are not only caused by metabolic events. A variety of stimuli and situations are powerful inducers of cortisol release from the adrenal cortex. As early as 1968, Mason reviewed more than 200 papers in which the psychoneuroendocrinology of the HPA axis was investigated. Summarizing different lines of evidence, Mason [1968] concluded that ‘(...) psychological influences are among the most potent natural stimuli known to affect pituitary-adrenal cortical activity.’

Situational characteristics experienced as novel, unpredictable, or uncontrollable have been shown to be closely associated with enhanced cortisol output. The majority of studies conducted with salivary cortisol as an indicator of stressful experience supports this viewpoint.
However, the literature on psychological stress and cortisol in saliva suggests that stress responses of the adrenal cortex are dependent on two critical psychological variables: (a) emotional ego involvement and (b) suspenseful anticipation of noxious events.

Both in laboratory experiments and in field studies cortisol levels in saliva mirrored physiological responses to psychological stress. Public speaking is one stress paradigm that affects the HPA axis. Bassett et al. [1987] have reported enhanced salivary cortisol levels in bank employees who had to deliver a 15-min lecture in front of an audience. Compared to a control day the steroid levels were increased in the anticipation period before the lecture and stayed elevated throughout the following 2 h. Unfortunately, the authors only obtained samples prior to and after the public lecture so no time course of the cortisol dynamics could be monitored. From a psychoneuroendocrinological point of view it would have been interesting to notice whether the peak cortisol values were reached before during or following the stressful task. Using a similar stress setting with sham-recorded public speaking as the demanding task we found that salivary cortisol levels were already highest after the 10-min preparatory phase [Lehnert et al., 1989]. This points to the central role of anticipation in the psychobiology of cortisol. A forthcoming event which implies potential negative consequences for the individual activates the HPA axis if the person perceives the situation as threatening. Multiple other examples for this effect can be found in the literature of salivary cortisol stress responses.

Ben-Aryeh et al. [1985] studied 51 women awaiting fine-needle aspiration for diagnosis of a lump in the breast. Samples collected by these women in the waiting room revealed higher salivary cortisol values compared to a control group of women examined in their homes. The authors suspected that the psychologically induced stress of prolonged anxiety and anticipation of a surgical procedure contributed to the steroid response.

Although neither unpredictable nor novel, flight operations like takeoffs and landings were shown to be relevant stressors for cortisol secretion from the adrenals. In transport aircraft pilots and copilots the level of salivary cortisol was monitored throughout long flights including multiple operations and it was apparent that increases in cortisol were time-matched with operations [Kakimoto, 1985]. Interestingly, copilots showed higher salivary cortisol responses than pilots throughout the course of this study, probably reflecting a higher uncontrollability in the former group.

Further evidence for a central role of anticipation of negative events influencing HPA activity is provided by studies on academic examinations. Jones et al. [1986] investigated the hormonal response to the first academic examination. At different times of day, 40 medical students obtained three saliva samples independently 3 days before the test and on the day of examination, respectively. Only the samples taken immediately prior to the test showed a significant enhancement compared to the control day. This result supports earlier findings of elevations of cortisol in saliva in response to examination stress [Stahl and Dörner, 1982]. Moreover, data from our laboratory suggest that inadequate coping and ruminating behavior may be positively correlated with salivary cortisol levels under these circumstances [Hellhammer et al., 1985].

The close interaction between psychological variables and salivary cortisol responses to a given situation is further underscored by data on the effect of venipuncture, which has been controversially discussed in the literature. Harris et al. [1988] compared salivary cortisol levels in 95 psychiatric inpatients before and after venipuncture and failed to find altered mean salivary hormone levels. In contrast, Hubert and Nieschlag [1988] observed 11 responders out of 16 subjects who were catheterized for LH-RH stimulation. The discrepant results reported here may be caused by at least three different reasons. Harris et al. [1988] did not segregate responders from nonresponders for statistical evaluation and cortisol levels were only determined once following venipuncture. If 20–30% of the subjects had shown a moderate response, t-test results would still indicate nonsignificant differences. Furthermore, no information on the time lag between venipuncture and sampling is provided. Secondly, at least some of the patients studied were on a tranquilizer up to 15 mg diazepam or equivalent daily. Most likely, these patients showed differences in affect and/or mood compared to the healthy controls studied by Hubert and Nieschlag [1988]. Thirdly, since all patients were inpatients for 2 weeks or longer they have experienced venipuncture several times before. All of these factors might have had an influence on cortisol values following venipuncture.

In an attempt to unravel psychosocial factors which determine the response/nonresponse after venipuncture we recently investigated 58 healthy blood donors and found a significant increase in salivary cortisol levels for the whole group after 10, 20, and 30 min after insertion of the catheter. We found 24 responders and 34 nonresponders according to the criterion of Van Cauter and
Refetoff [1985] as transformed to saliva conditions [Alloïo et al., 1986]. Analysis of variance indicated that the factor 'experience of the situation' accounted for a considerable amount of total variance. Those subjects who donated blood for the first time showed the highest salivary cortisol responses.

Moreover, we found a significant interaction of sex and repression/sensitization. Women scoring high on a sensitization scale and men with high repression scores had increased cortisol values after venipuncture while the two remaining groups of the $2 \times 2$ design did not respond to the 'treatment'. This indicates that venipuncture has no impact per se on the HPA axis in the human. It is the interplay of situational factors like novelty and personality traits like anxiety or sensitization that may lead to the respective endocrine responses. Again, in our view anticipation of threatening or noxious events associated with the procedure of venipuncture is responsible for the psychoendocrine reaction observed in some of the subjects investigated.

Yet another psychological variable is crucial for any adrenal stress reactions to psychological stimulation. Data from several studies applying low intensity laboratory stressors suggest that emotional egoinvolvement is a prerequisite for salivary cortisol responses to occur following nonnoxious challenges. The presentation of stressful film sequences has frequently been employed to elicit changes in physiological parameters including salivary cortisol. Hellhammer et al. [1986] were able to show that suspenseful anticipation (as a consequence of egoinvolvement) was associated with increases of cortisol in saliva while tension alone did not evoke secretion of cortisol. Watching the movies 'Psycho' and 'Goldfinger' on two separate occasions the subjects showed significantly elevated cortisol levels in the 'Psycho' film compared to 'Goldfinger' where cortisol values continuously fell due to circadian rhythm. The interaction of personality variables and cortisol responses under film stimulation was further investigated in a study with children [Kirschbaum et al., 1989a]. Salivary cortisol patterns were indistinguishable between stress and control film when all subjects were treated as a single group. However, a distinct cortisol response was observed separating children with high scores on a trait anxiety scale from children with low scores. The latter group had decreasing salivary cortisol levels in response to both films and the opposite picture emerged with the high-anxiety subjects. Hubert and de Jong-Meyer [1989] also found a significant correlation between salivary cortisol responses to a suspenseful movie and state anxiety. However, they did not find a similar correlation with trait anxiety. In line with these findings there is some evidence that such a cortisol response can be blocked by diazepam [Hellhammer et al., 1988].

A study of considerable interest in terms of the interaction of emotional egoinvolvement and cortisol stress responses has been finished just recently. Wittling and Pflüger [in press] presented a film of 3 min duration showing electroconvulsive treatment of psychiatric patients to either the right or the left hemisphere of healthy volunteers. They observed enhanced cortisol levels when the film was presented to the right hemisphere (controlling emotional processes) and decreasing values after projection to the left hemisphere. Upon replication, these results would strongly support the hypothesis that a cortisol response under psychological stress is dependent on emotional processing of stressful stimuli.

We suspect that besides situational variables (like unpredictability, uncontrollability and novelty) the individual's perception of a situation as being potentially detrimental in concert with its emotional processing is the major trigger of cortisol secretion in response to stressful psychological stimulation. The more novel, uncontrollable, or unpredictable the situation, the greater the hormone output will be. (Of course, we do not assume that the relation between stimulation intensity and magnitude of cortisol response is linear!) This implies that with repeated exposure to the same psychological stimulation – lacking significant negative consequences – an initial cortisol response will gradually habituate.

**Methods**

The acceptance of any new methodological approach to the assessment of hormones in a given clinical or research environment depends on the ease of sampling, the storage conditions of specimens, and the reliability of available assays for analysis. Given that these criteria are satisfactorily fulfilled, the economy of the new technique has to be advantageous to the conventional methods in use. The measurement of cortisol in saliva does meet all the criteria mentioned above compared to plasma or serum cortisol determination. Thus, in most instances adrenal activity is easier and cheaper to assess in the human if the method of salivary cortisol is employed. In this chapter we will briefly describe and evaluate methodological issues associated with the salivary cortisol technique.
Sampling and Storage of Saliva

Saliva samples can be obtained by different means. Most frequently subjects are asked to simply salivate into disposable tubes or small plastic containers. Although it is the cheapest way of sampling it bears a number of disadvantages. Some subjects show an emotive bias against sight ing saliva running out of their mouths. It can be rather unhygienic and more difficult for the technician if samples have to be transferred into smaller tubes for centrifugation. Finally, in field studies or ambulatory monitoring subjects may find it unacceptable to gather saliva this way. The use of a device especially designed for saliva sampling circumvents the above-mentioned problems. The 'Salivette' (Sarstedt Inc., Rommeldorf, FRG) mainly consists of a sterilized cotton swab, a small beaker, and a simple plastic tube [Hellhammer et al., 1987]. Since flow rate does not influence cortisol levels [Hiramatsu, 1981; Guéchet et al., 1982; Vining et al., 1983a; Bustamante and Crabbe, 1984], a saliva sample is quickly obtained by chewing gently on the cotton swab. The stimulation of saliva flow rate by chewing ensures that the resulting samples have sufficient volume to allow multiple analyses. The cotton swab is left in the mouth for 30–60 s and is subsequently put into the small beaker. After centrifugation at 3,000 rpm for 2 min 0.5–1 ml of saliva can be pipetted out of the disposable tube. The same device is available with citric acid as a flow rate stimulator, but this might interfere with the biochemical assay used for cortisol quantification. In their sophisticated review, Vining and McGinley [1986] suspected that steroids could unsystematically adhere to cotton material, thus leading to erroneous hormone values. While this is true for testosterone we found no impact on cortisol values when the cotton swab was used (unpublished observation). In several investigations we found the Salivette a useful device for saliva sampling in subjects from 4 to 70 years of age. With a brief introduction these subjects find no problems in obtaining samples independently at home, at work, in the hospital, or in the research laboratory.

If saliva from younger children is to be sampled other techniques should be applied. After stimulation of saliva flow by a small citric acid crystal put on the child's tongue, mothers or nurses can aspirate sufficient saliva from the floor of the mouth by means of a pipette [Hiramatsu, 1981], a wide-bore plastic tube attached to a syringe [Riad-Fahmy et al., 1982] or a disposable mucus extractor [Price et al., 1983].

The storage of saliva samples is uncomplicated. Kahn et al. [1988] found no alteration of salivary cortisol values after storage of samples at room temperature for 2 weeks before analysis. In addition, there was no detectable influence of centrifugation before storage. Recently, we could show that after storage of samples at ambient temperature salivary cortisol concentrations were not altered for up to 30 days [Kirschbaum and Hellhammer, unpublished data]. In this experiment we observed slowly decreasing cortisol values when samples were stored at room temperature for more than 20 days possibly indicating a breakdown of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase. However, the differences did not reach statistical significance. Samples can thus be conveniently stored at the subjects' homes for several days and there is no need for subsequent shipment to the laboratory on dry ice.

Assays of Cortisol in Saliva

Cortisol is present at low concentrations in saliva. Reliable assessment of salivary cortisol thus calls for a sensitive analytical method. Although alternative techniques are available, RIA is still the method used most frequently for the determination of the steroid.

Radioimmunoassays

Walker et al. [1978] were the first to develop a highly sensitive RIA which allowed the duplicate measurement of salivary cortisol from a 20-μl sample. Following this report, several research groups worked out their own 'in-house' RIAS and successfully employed the method in their studies [Stahl et al., 1978; Guéchet et al., 1981; Umeda et al., 1981; Poland and Rubin, 1982; Vining et al., 1983b; Bustamante and Crabbe, 1984; Jones et al., 1986].

However, assays developed in a research laboratory are usually not commercially available and reagents will rarely be forwarded. Wherever 'in-house' reagents are unavailable, adaptations of commercial assay kits designed for serum or plasma cortisol analyses were used. Several modifications of such assays have been published recently [Al-Ansari et al., 1982; Silver et al., 1983; Vittek et al., 1983; Woolston et al., 1983; Ansseau et al., 1984; Burke et al., 1985; Hanada et al., 1985; Hellhammer et al., 1985; Fibiger et al., 1986; Al-Hakiem and Abbas, 1987; Simon et al., 1987; Kirschbaum et al., 1989b].

In general many of these assays allow for reliable determinations of salivary cortisol. However, technical
and/or economical requirements limit the number of adequate kits. First of all, the dynamic range of the assay used is crucial for the reliability of results. The quotient of \( B/B_0 \) (0–standard/highest cortisol standard) indicates the vulnerability of the assay to inaccurate sample processing. The lower the quotient the less precise the analysis will be. In some cases a second analysis of samples is required (e.g. due to inaccuracy of the first assessment), thus one should choose an assay employing a small sample volume. Due to differences in assay sensitivity, between 50 and 400 μl are used for a duplicate analysis of saliva with these adaptations.

An outstanding feature of salivary cortisol measures is the opportunity of sampling at any desired frequency even outside the laboratory. In the past, only few research groups were able to take advantage of this feature because of the high costs for analytical material. Upon adaptation of serum cortisol kits without coated tubes costs for reagents can be cut down significantly. Since fewer antibodies and less tracer is needed in a sensitive assay a single salivary cortisol determination can be obtained at 25% of the price for a single serum analysis [Kirschbaum et al., 1989b]. Given a certain budget for biochemical reagents it is now possible to analyze samples from larger groups of subjects and/or at shorter intervals, thus increasing the reliability of results.

**Alternative Biochemical Analyses of Salivary Cortisol**

A number of disadvantages are associated with cortisol assessment by RIA, for instance radioactive waste disposal or instability of tracer. Various biochemical procedures have been elaborated to circumvent these obstacles including high pressure liquid chromatography [Rose and Jusko, 1979; Saito et al., 1981], polarization fluoroimmunoassay [Al-Ansari et al., 1983], enzyme immunoassays [Hubl et al., 1984, 1988], and chemiluminescence immunoassays [Strasburger et al., 1987; Hubl et al., 1988]. Unfortunately, none of the alternative techniques mentioned above is commercially available to date.

**Dynamic Tests of HPA Axis Activity Employing Salivary Cortisol Measures**

Several pharmacological tests are routinely performed to investigate the functioning of the HPA axis and its feedback loops. Most clinical endocrinologists still use plasma or serum cortisol determinations for this purpose despite obvious advantages of salivary samples for steroid concentration assessment. It is only recently that cortisol in saliva entered routine use in HPA testing [Allofio et al., 1986]. In the following chapter we briefly describe three pharmacological tests most frequently employed in the diagnosis of HPA axis functioning and discuss its application with saliva as the alternative to blood monitoring.

**Corticotropin-Releasing Hormone Challenge**

Following analysis of the 41-amino acid residue and subsequent synthesis of corticotropin-releasing hormone (CRH) [Vale et al., 1981], the main corticotropin-releasing factor rapidly became a standard HPA challenge test in clinical endocrinology and psychiatry [Gold et al., 1984, 1986; Schürmeyer et al., 1984]. A bolus i.v. injection of 1 μg/kg or 100 μg of synthetic h-CRH is routinely used as the standard CRH challenge test representing the lowest dose which gives a maximal cortisol response in healthy subjects. Since the peptide does not readily cross the blood-brain barrier the CRH test is suited for studying the response of the pituitary corticotrophs and the responsibility of the adrenal cortex to ACTH.

Only few researchers measured cortisol in saliva as an equivalent to plasma cortisol values so far. They could show that salivary cortisol responses to CRH parallel the respective responses in plasma. Peak values are obtained between 30 and 45 min after injection [Kahn et al., 1988; Kirschbaum et al., 1989b; Lehnert et al., 1989]. However, the magnitude of increase (400–600% from baseline levels) appears to be more pronounced in saliva compared to plasma. Similar findings have been obtained by ACTH stimulation (see below). The typical time course of salivary cortisol responses to injection of 100 μg h-CRH is shown in figure 5. For use with saliva, samples are obtained 5–10 min before and at 10 to 15-min intervals after injection of CRH over a period of 60–180 min.

**ACTH Test of Pituitary Reserve**

ACTH analogues are administered in the clinical investigation of pituitary reserve. In contrast to the CRH challenge test, salivary cortisol responses to ACTH have been measured by numerous researchers [Katz and Shannon, 1964; Walker et al., 1978; Hiramatsu, 1981; Umeda et al., 1981; Al-Ansari et al., 1982; Riad-Fahmy et al., 1982; Vining et al., 1983b; Brooks and Brooks,
the central nervous system. If the organism secretes a considerable amount of cortisol despite a high dexamethasone level, an abnormal functioning of the HPA axis is diagnosed. Especially in Cushing’s disease and endogenous depression patients are found to be escaping from dexamethasone suppression within 17 h. In the typical dexamethasone suppression test (DST) protocol subjects take 1 mg dexamethasone at 11 p.m. and cortisol values are determined from samples at 8 a.m. the following morning.

Several workers applied parallel cortisol determinations in plasma/serum and saliva to study the responses to overnight dexamethasone suppression [Al-Ansari et al., 1982; Poland and Rubin, 1982; Vining et al., 1983b; Anseau et al., 1984; Hanada et al., 1985]. Although rather few subjects were investigated, salivary cortisol levels appeared to reliably reflect suppression in blood. In a first large-scale clinical study Cook et al. [1986a] obtained pre- and postdexamethasone saliva and plasma cortisol levels from 162 routine psychiatric admissions. A comparison of DST results revealed an identical utility of both specimens, which was further supported by recent data from Kahn et al. [1988].

Applying the DST to a different population Hoshino et al. [1987] found abnormal DST salivary cortisol responses and circadian rhythms in autistic children. In poorly developed cases they observed abnormalities of HPA functioning more frequently than in higher-developed ones. This finding could well stimulate further investigations of HPA functioning in populations yet unknown to display abnormal patterns and extend the application of the convenient measure of cortisol in saliva as a clinical tool.

**Practical Issues**

Several practical problems are always encountered where hormone concentrations serve as the dependent variable. In this final chapter we intend to pinpoint some of the most significant questions associated with salivary cortisol measures and to summarize the answers to these questions given in the literature.

**When Are Salivary Cortisol Measures ‘Useful’?**

Obviously, one of the most ‘useful’ applications of salivary cortisol assessment is the investigation of HPA axis functioning in patients. Under several physiological
conditions with elevated CBG levels the determination of cortisol in saliva is a more valid estimation of the biologically active hormone fraction than total plasma cortisol. Moreover, in infants and children the cortisol system should be determined from saliva samples.

Furthermore, salivary cortisol can be a sensitive measure in stress research. Primarily in short-term laboratory and field experiments cortisol in saliva may be assessed as a convenient and reliable parameter of endocrine stress responses. In studies using psychological stressors it appears to be crucial for any cortisol response that the subjects get involved and anticipate potential negative consequences of the situation. Unlike short-term stress, there are no convincing data available on chronic stress effects on cortisol in saliva. Such studies are needed to elucidate whether the human cortisol system adapts to prolonged stress or rather elicits physiological alternations as it has been shown in rodents [Sapolsky et al., 1986]. The latter would underscore the possible impact of stress in the onset and course of diseases with special focus on glucocorticoids as mediators.

What Is a Cortisol Response?

Studies of cortisol responses to any treatment require appropriate baseline values which allow for conclusions as to which extent the subjects show increased adrenal activity following stimulation. Depending on the time span of the investigation samples must be obtained at different intervals. If a stress-related alteration of the circadian hormone profile is to be studied samples should be taken at 8–9 a.m., 11–12 a.m., 3–4 p.m., and 8–10 p.m. Within these periods relatively small changes in unstimulated cortisol values are observed, thus a satisfactory estimation of the circadian rhythm can be acquired. Baseline or control samples should be obtained either before or 1–3 weeks after the stressful period at identical time points. Since there is considerable day-to-day intraindividual variation in cortisol concentrations a second control circadian profile may be necessary.

In short-term stimulation experiments it is even more important to take into consideration the circadian rhythmicity of cortisol. A peak concentration measured at 11 a.m. may not yield significant differences when compared to a single ‘baseline’ value taken at 8 a.m. Although the subject did respond to the stimulation the effect would have been masked under this condition. In an earlier paper we proposed the ‘practical baseline’ method to handle the problem adequately [Hellhammer et al., 1987]. Following this method a cortisol response can be revealed by measuring the deviation of cortisol from an overall decreasing practical baseline, which is defined by a negative linear trend between pre- and post-measures. In conjunction with this method responders may then be classified by the criterion of Van Cauter and Riefstoff [1985] adapted for saliva cortisol measures: an increase of cortisol in saliva of at least 2.76 nmol/l in two or more consecutive measurements and at least 15% from a previous value represents a secretory episode [Alloio et al., 1986].

What Is the Time Latency between Stimulation and the Peak Cortisol Concentration?

Pharmacological, physical, or psychological stimulation of the adrenal cortex results in transient rises of cortisol concentrations in bodily fluids. Peak hormone levels are monitored after different time latencies depending on the intensity and (probably) on the nature of the stimulation. Following the standard CRH challenge of 100 μg or 1 μg/kg body weight, cortisol in saliva peaks at 30–45 min [Kahn et al., 1988; Kirschbaum et al., 1989b; Lehnert et al., 1989]. Supraphysiological pharmacological challenges of the cortisol system with synthetic ACTH (Synacthen) lead to a yet prolonged increase of salivary cortisol levels for at least 60–90 min after drug injection [Landon et al., 1984; Peters et al., 1984; Alloio et al., 1985]. Obviously, there is a dose-response relationship for the time latency between onset of stimulation of the adrenals and peak hormone response. Investigating the CRH challenge test, Schürmeyer et al. [1984] observed a proportional increase in time latency with increasing doses of CRH. In their experiments plasma cortisol peaked between 5 and 60 min following injections of CRH at doses between 0.01 and 5 μg/kg. As for the time latency following psychological stimulation, cortisol in saliva appears to peak 20–30 min after the onset of a rather mild laboratory stress [Fibiger et al., 1986; Hubert and de Jong-Meyer, 1989; Kirschbaum et al., 1989a]. These results suggest a CRH release of rather moderate quantity in response to psychological laboratory stressors. However, more detailed studies are needed to elucidate the relationship between intensity and nature of the stimulus and the time lag between onset of stimulation and peak cortisol levels in saliva.
Which Psychobiological Topics Need to Be Investigated in the Future Using Salivary Cortisol Measures?

As we pointed out in this article, the method of cortisol determination in saliva appears to gain increasing interest in psychobiological research due to several advantages over blood analyses. Since anyone who has access to a gamma counter can now perform analyses of salivary cortisol in his own laboratory at reasonable costs, this method could help to illuminate some of the basic problems in the psychoendocrinology of cortisol.

One question of particular interest is whether the time courses of salivary cortisol responses to pharmacological, physical, or psychological stimuli are different from each other. If so, investigations on the pathways of these responses would be challenging. Closely related to this topic is the question whether intrindividually the magnitude of response to one of these stimuli can predict the subject's reaction to another stimulus. For example it can be hypothesized that the susceptibility of the adrenal cortex to moderate pharmacological stimulation may reflect the individual's 'cortisol responsiveness' to psychological stressors as well. Furthermore, habituation phenomena in cortisol responses have received only little attention in the past. What are the mechanisms and the rationale of habituated or decreased cortisol responses to repeated stimulation? Do all stimuli encountered in daily life lead to non-reaction in the end or are there situations and/or personality factors which prevent habituation?

Of course, this is only a short list of questions and problems associated with cortisol assessment in psychobiology. Although many more may follow, we now hold the tools in our hands to proceed more quickly in unravelling the psychobiology of the human cortisol system.

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