

A dose–response study of salivary cortisol after dexamethasone suppression test in Cushing’s disease and its potential use in the differential diagnosis of Cushing’s syndrome

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Summary

OBJECTIVE A dose–response study with different doses of dexamethasone (dex) to assess the corticotropic resistance in Cushing’s disease (CD) using salivary cortisol as an end point has not yet been evaluated. We also reported our experience with salivary cortisol compared to plasma cortisol determination during dex suppression test (DST) and after ovine corticotrophin release hormone (oCRH) test in the differential diagnosis of Cushing’s syndrome (CS).

DESIGN We studied 46 patients with CS, including 28 patients with CD, 16 with adrenal disease and two with occult ectopic adrenocorticotrophic hormone (ACTH) tumours. Salivary cortisol was compared to plasma cortisol and ACTH during a DST 2 mg for 2 days, 8 mg for 2 days and 24 mg for 1 day, and after oCRH test.

RESULTS We observed a dose-dependent suppression of salivary cortisol, plasma cortisol and ACTH in CD patients. Salivary cortisol presented a higher percentage of suppression than plasma cortisol: 42% vs. 15% ($P < 0.002$), 82% vs. 67% ($P < 0.002$) and 90% vs. 83% ($P < 0.03$) after 2, 8 and 24 mg/day dex, respectively. The lowest percentage of suppression was observed for plasma ACTH. The parallelism of these lines identified that the criterion of 65% suppression of salivary cortisol corresponding to 50% suppression of plasma cortisol after 8 mg/day for 2 days is consistent with CD. The sensitivity and specificity using 50% suppression

for plasma cortisol were 81% and 83%, respectively, for 8 mg DST. Using the criterion of 65% suppression of salivary cortisol, the sensitivity and specificity were 86% and 100%, respectively, for 8 mg DST. After oCRH test the sensitivity and specificity were 86% and 91%, respectively, for ACTH, 100% and 64%, respectively, for plasma cortisol and 93% and 91%, respectively, (20% of increment) or 86% and 100%, respectively, (35% increment) for salivary cortisol.

CONCLUSION In conclusion, salivary cortisol presents more profound suppression than plasma cortisol or ACTH in a dose–response pattern after different doses of dex in patients with CD. In addition, our data suggest that measurement of salivary cortisol might improve the DST as compared to plasma cortisol in the differential diagnosis of CS.

Salivary cortisol reflects free cortisol in the plasma levels (Luthold *et al.*, 1985; Raff, 2000) and its measurement at midnight and after the overnight 1-mg dexamethasone (dex) tests were demonstrated to be as sensitive as the conventional gold standard tests for screening Cushing’s syndrome (CS) in adults and children (Raff *et al.*, 1998; Castro *et al.*, 1999; Martinelli *et al.*, 1999). The differential diagnosis of CS requires several pituitary–adrenal axis tests to determine the aetiology, being the high-dose dexamethasone suppression test (DST) and the oCRH, the most commonly tests used in order to differentiate adrenocorticotrophic hormone (ACTH)-dependent causes of CS (Crapo, 1979; Orth, 1995; Newell-Price *et al.*, 1998). Bilateral simultaneous inferior petrosal sinus catheterization (IPSS) with sampling for ACTH is now accepted as part of the investigation protocol of ACTH-dependent CS in adults (Oldfield *et al.*, 1991) and in children (Lienhardt *et al.*, 2001) but its use is available in only a small number of specialist centres.

DSTs are based on the partial resistance to glucocorticoid-negative feedback inhibition observed in corticotroph adenomas. The original report by Liddle (1960; 8 mg dex/day for 2 days) used urinary 17-hydroxycorticosteroids (17-OHCS) measurement and, thereafter, this test has been performed with plasma cortisol or urinary free cortisol (UFC) measurement (Flack *et al.*,

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1992). Further studies have hypothesized that a very high dose of dex (24 or 32 mg for 1 day) might produce more effective suppression of cortisol secretion in Cushing's disease (CD), resulting in clear superiority to the high-dose DST (Al-Saadi *et al.*, 1998). The daily dose of dex in the high DST as described by Liddle is traditional but arbitrary. There is no dose-response study using salivary cortisol after different doses of dex to overcome the glucocorticoid relative resistance in CD.

In the present study we examined the performance of salivary cortisol after different doses of dex compared to plasma cortisol and plasma ACTH determination in patients with ACTH-secreting pituitary tumours. In addition, the use of salivary cortisol has not yet been evaluated as an alternative end point in the DST and oCRH tests for differential diagnosis of CS. Therefore, we also reported our experience with salivary cortisol compared to plasma cortisol determination during DST and after oCRH test in the differential diagnosis of CS.

Patients and methods

Forty-six patients with CS (five men and 41 women) were investigated between 1994 and 2000 in the University Hospital of the School of Medicine of Ribeirao Preto, after obtaining written informed consent. Twenty-eight of them presented with CD, 16 with adrenal disease (including 13 patients with adrenal tumour, two with primary pigmented nodular adrenocortical dysplasia and one with adrenocortical nodular hyperplasia) and two with occult ectopic ACTH-producing tumours. In addition to the clinical features of chronic hypercortisolism, the diagnosis of CS was established, independently of salivary cortisol measurement, by standard tests of pituitary-adrenal function including plasma ACTH levels, low-, high- and very high-dose DST, oCRH test and IPSS with oCRH, when appropriated (Findling & Doppman, 1994; Newell-Price *et al.*, 1998), in addition to image studies. There was no evidence of an apparent tumour on computed tomography (CT) or magnetic resonance imaging (MRI) in patients with the occult ectopic ACTH syndrome. The diagnosis of corticotroph adenomas was confirmed by curative transsphenoidal surgery or positive tissue pathology.

Low-, high- and very high-dose DST

In order to compare the dose-response of salivary cortisol, plasma cortisol and ACTH to different doses of dex, 28 patients with CD were evaluated before and after oral DST: 2 mg/day (0.5 mg every 6 h for 2 days), 8 mg/day (2 mg every 6 h for 2 days) and 24 mg/day (6 mg every 6 h for 1 day), administered sequentially and always in ascending order of doses. We also performed the low-, high- and very high-dose DST in two patients with ectopic ACTH-secreting tumours. Patients with primary adrenal tumours ($n = 16$) had already been identified

as having ACTH-independent CS and would not be subjected to 8 mg or 24 mg DST. However, we tested these patients as negative controls.

Blood for plasma ACTH and cortisol assays and saliva samples for cortisol determination were collected at 09:00 h at the beginning and at the end of each test from hospitalized patients.

oCRH test

Twenty-five patients (14 patients with CD, nine with adrenal disease and two with ectopic ACTH syndrome) and six control subjects were submitted to oCRH test. After an overnight fast, oCRH test (1 µg/kg; Bachem, Torrance, CA, USA) was given as an intravenous injection at 09:00 h. Blood samples for plasma ACTH and cortisol levels were obtained at zero and 15, 30, 45, 60, 90 and 120 min, and salivary samples for cortisol assay were obtained before and every 30 min after oCRH injection until 120 min. An increment of 20% baseline levels for plasma cortisol and 35% for ACTH after oCRH was regarded as significant stimulation (Nieman *et al.*, 1993; Newell-Price *et al.*, 1998).

Assays

Salivary cortisol was determined by a previously described radioimmunoassay (RIA) in 25-µl samples of saliva (Santiago *et al.*, 1996; Castro *et al.*, 1999). The assay sensitivity was 1.7 nmol/l. The intra- and interassay coefficients of variation (CVs) were 5.5% and 11%, respectively. Plasma cortisol and ACTH levels were determined by RIA, as previously described (Moreira *et al.*, 1993). The sensitivity and the intra- and inter-assay CVs were 33 nmol/l, 4.5% and 11% for plasma cortisol, and 2 pmol/l, 6% and 16% for plasma ACTH, respectively. All samples obtained from each subject were analysed in duplicate in the same assay.

Statistical analysis

Data were expressed as mean ± SEM. We assumed that the percentage of decrease of salivary cortisol, plasma cortisol and ACTH after DST in CD patients depends on the dex dose and to perform the subsequent analysis we used the logarithm to the base 2 of the dex dose (dex log₂). To accommodate within subject dependence of the repeated measures, Hotelling's T₂ statistics have been used to test for parallelism and equality of mean dose-response profiles (Zar, 1984). For both high- and very high-dose DST, a reduction of plasma cortisol of 50% or more from baseline was consistent with CD, as generally accepted. Using the dose-response DST lines, we identified the criterion for salivary cortisol suppression. The Wilcoxon paired test or paired *t*-test were also used when appropriated. Significance was assumed when $P < 0.05$.

Results

Low-, high- and very high-dose DST

The absolute levels of plasma ACTH and cortisol and salivary cortisol were available before and after 2, 8 and 24 mg/day DST in patients with CD. Plasma ACTH levels were 15.6 ± 2.2 , 11.2 ± 1.3 , 6.6 ± 0.9 and 5.1 ± 0.7 pmol/l; plasma cortisol were 644 ± 48 , 532 ± 73 , 196 ± 45 and 101 ± 20 nmol/l; and salivary cortisol were 39 ± 5.6 , 25 ± 5.6 , 5.6 ± 1.7 and 2.8 ± 0.2 nmol/l, respectively, before and after 2-, 8- and 24-mg dex tests (Fig. 1a).

Figure 2 shows the mean percentage of suppression from baseline of plasma ACTH, plasma cortisol and salivary cortisol in CD patients. All CD patients presented a higher percentage of salivary cortisol than plasma cortisol suppression: 42% vs. 15% ($P < 0.002$), 82% vs. 67% ($P < 0.002$), 90% vs. 83% ($P < 0.03$) after 2, 8 and 24 mg/day DST, respectively. There was a positive correlation between salivary and plasma cortisol ($r = 0.85$, $P < 0.0001$) at low, medium and high concentrations. The lowest percentage of suppression was observed for plasma ACTH: 0%, 40% and 49% after 2, 8 and 24 mg/day DST, respectively.

There was a negative dose-response over the dex doses for salivary cortisol, plasma cortisol and ACTH levels ($P < 0.001$). We also observed that there was a \log_2 dose-dependent suppression of salivary cortisol, plasma cortisol and plasma ACTH ($P <$

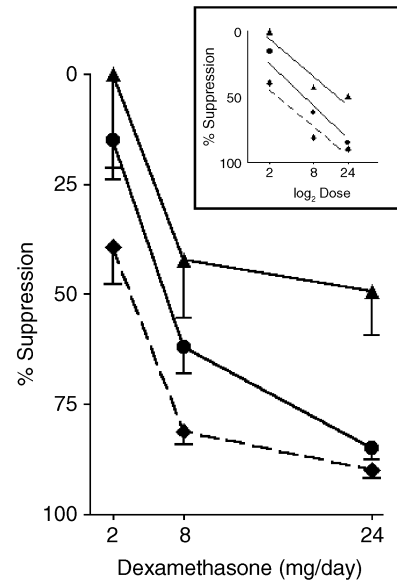


Fig. 2 Percentage of suppression of plasma ACTH (\blacktriangle), plasma cortisol (\bullet) and salivary cortisol (\blacklozenge) levels in a dose-response pattern after different doses of dexamethasone in patients with Cushing's disease ($n = 28$). The baseline levels are defined as 0%. Upper insert: Data after logarithmic transformation of dexamethasone dose to the base 2 (\log_2).

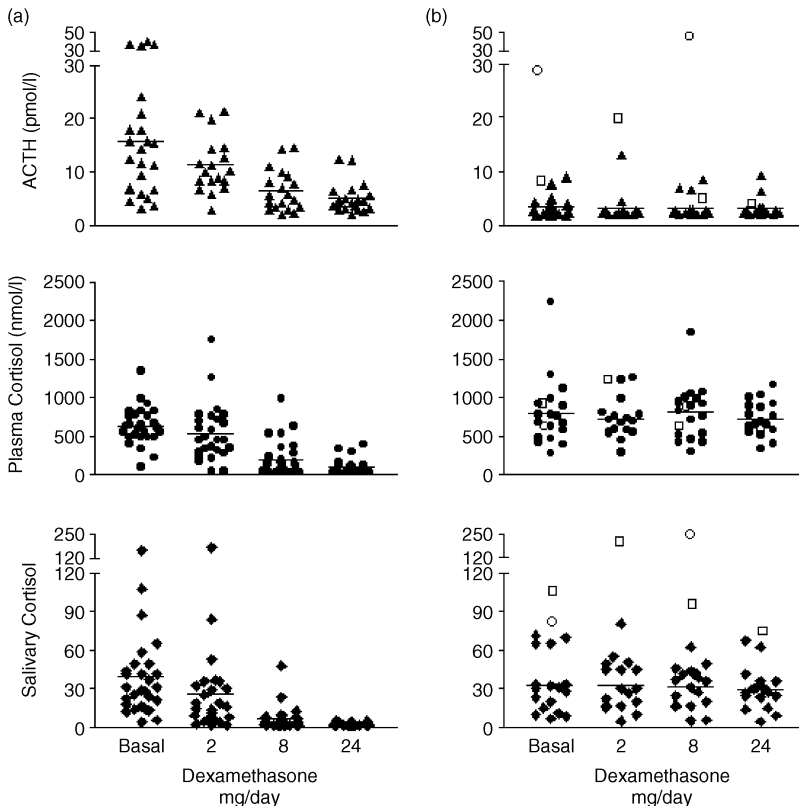


Fig. 1 Plasma ACTH, plasma and salivary cortisol levels before and after different doses of dexamethasone. (a) Patients with Cushing's disease ($n = 28$); (b) other forms of Cushing's syndrome ($n = 18$). Open symbols indicate occult ectopic ACTH syndrome.

0.05). There is no evidence that the lines of percentage of suppression of salivary cortisol, plasma cortisol and plasma ACTH were not parallel ($P > 0.05$, Fig. 2, insert). The profile of salivary cortisol suppression line was not coincident with plasma cortisol and plasma ACTH suppression lines. However, plasma cortisol and plasma ACTH suppression lines presented coincident profiles. In addition, based on the parallelism, we identified that the criterion of 65% suppression of salivary cortisol corresponding to 50% suppression of plasma cortisol after dex 8 mg/day for 2 days as consistent with CD.

In patients with adrenal tumours, plasma ACTH levels were 3.1 ± 0.4 , 2.3 ± 0.1 , 2.4 ± 0.1 and 2.5 ± 0.1 pmol/l; plasma cortisol were 812 ± 106 , 728 ± 62 , 812 ± 84 and 728 ± 56 nmol/l; and salivary cortisol were 34 ± 5.3 , 34 ± 4.8 , 31 ± 3.6 and 31 ± 3.9 nmol/l, respectively, before and after 2-, 8- and 24-mg dex tests (Fig. 1b). The two patients with occult ectopic ACTH secretion presented high basal ACTH levels (29 and 9 pmol/l), plasma cortisol (605 and 918 nmol/l) and salivary cortisol levels (91 and 151 nmol/l) and no suppression of salivary or plasma cortisol was observed after DST.

CD and other causes of CS presented similar baseline of plasma cortisol and salivary cortisol levels. CD patients as a group showed suppression of plasma cortisol and salivary cortisol

levels after 8 mg dex test ($P < 0.05$). Twenty-two out of 27 patients with CD showed at least 50% suppression of plasma cortisol. All patients with CD showed at least 50% suppression of salivary cortisol. Fifteen out of 18 patients with other causes of CS did not show 50% suppression of plasma cortisol after 8 mg DST, while 16 out of 17 did not show 50% suppression of salivary cortisol. We also used 65% of suppression as a cut-off for salivary cortisol after 8 mg DST for the diagnosis of pituitary disease. Twenty-four out of 28 CD patients showed at least 65% suppression of salivary cortisol after the 8-mg DST. However, no patient with other causes of CS showed 65% suppression of salivary cortisol.

The sensitivity and specificity of the 8 mg DST using 50% suppression as a cut-off were 81% and 83% for plasma cortisol, and 100% and 94% for salivary cortisol. Using 65% suppression of salivary cortisol as a criterion, sensitivity and specificity were 86% and 100% for 8-mg DST. Plasma and salivary cortisol had a greater sensitivity after 24 mg DST (92% and 100%) compared to 8 mg DST.

oCRH test

Figure 3 presents the mean of the absolute values (Fig. 3a) and the percentage of increase (Fig. 3b) of plasma ACTH, plasma

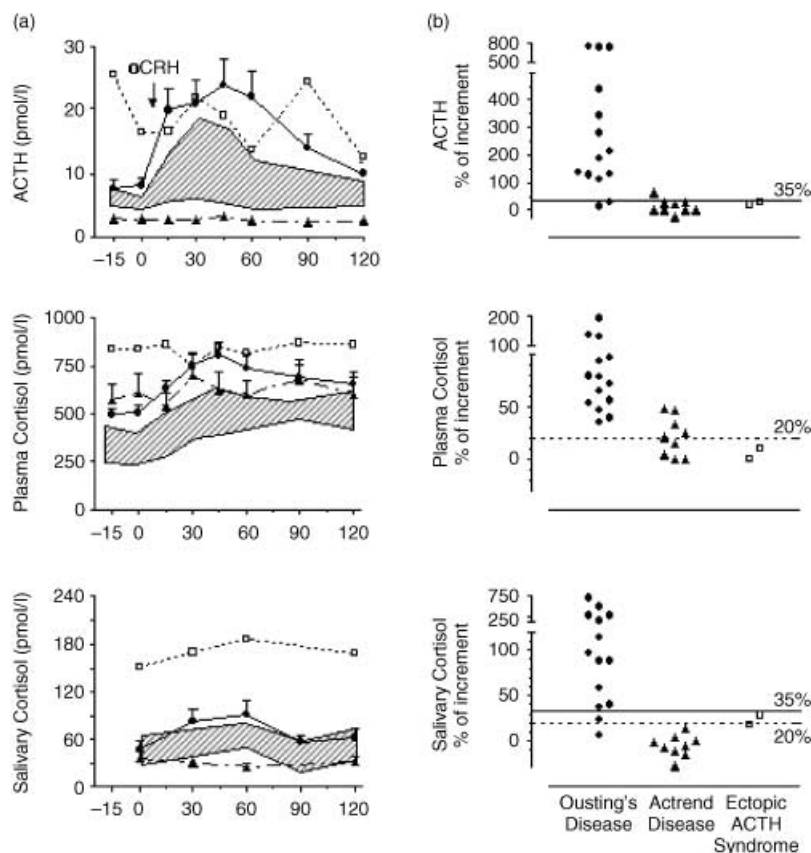


Fig. 3 (a) Salivary cortisol, plasma cortisol and ACTH responses to oCRH in control subjects (hatched area) and in patients with Cushing's disease (●), adrenal causes of Cushing's syndrome (▲) and occult ectopic ACTH syndrome (□). (b) Individual values of percentage of increment from baseline of salivary cortisol, plasma cortisol and ACTH after oCRH.

cortisol and salivary cortisol after oCRH in the control, CD, occult ectopic ACTH syndrome and adrenal causes of CS groups. Patients with ectopic ACTH syndrome are presented as a mean without standard deviation as we studied only two patients. In the normal subjects, plasma ACTH peaked at at least 35% ($183 \pm 28\%$) of the basal level after oCRH at 15–30 min, ranging from 39 to 384%. Plasma cortisol peaked at at least 20% of the basal level ($119 \pm 32\%$) at 45–90 min, ranging from 53 to 237%, whereas salivary cortisol peaked at 60–90 min ($197 \pm 76\%$), ranging from 54 to 500%. The peak response in relation to the basal levels of plasma ACTH, plasma cortisol and salivary cortisol after oCRH test in CD patients were $303 \pm 71\%$, $84 \pm 12\%$ and $173 \pm 52\%$, respectively.

All but two CD patients showed an increase in ACTH concentrations of at least 35% above mean basal values after oCRH, while all patients showed an increase in plasma cortisol concentrations of at least 20% above the mean basal values after oCRH. Ten out of 11 patients with other causes of CS did not show a 35% increase of ACTH, and four of these patients presented a 20% increase of plasma cortisol after oCRH. These data resulted in different sensitivities and specificities for plasma ACTH (86% and 91%) and plasma cortisol (100% and 64%) after the oCRH test. The sensitivity and specificity for salivary cortisol using 20% or 35% increase from basal levels were 93% and 91% or 86% and 100%, respectively.

Discussion

The present study focused on the measurement of salivary cortisol compared to plasma cortisol and ACTH, after different doses of dex in order to assess the glucocorticoid partial resistance observed in corticotroph adenomas. The analysis showed a dose–response relationship for plasma ACTH, plasma cortisol and salivary cortisol and the administered dex doses, and also a parallelism of the responses to dex among these three variables in CD patients. As previously described, the dose–response relationship of ACTH and plasma cortisol in CD was shifted to the right when compared to the response observed in normal subjects (Wolfsen & Odell, 1980; Bertagna *et al.*, 1995).

In the present study, we obtained further data showing that this relationship is also evident for salivary cortisol. In addition, we also demonstrated that salivary cortisol presents more profound suppression than plasma cortisol and ACTH in a dose–response pattern after different doses of dex in CD. The reduction of plasma ACTH determined a proportionally decline in plasma cortisol and a greater reduction in salivary cortisol levels. This phenomenon indicates the existence of a dex dose-dependent processing chain from pituitary to adrenal glands and from plasma cortisol to salivary cortisol. The reasons for the greater salivary cortisol suppression by dex are not clear. As previously suggested for UFC by Cope & Black (1959), this is probably

attributable to the binding of plasma cortisol by cortisol-binding globulin, limiting the amount of free diffusible cortisol. Therefore, cortisol in saliva falls much more rapidly than does the total plasma cortisol concentration and can more completely mirror cortisol secretion changes in suppression tests. The data also allowed us to verify that the very high dose of dex (24 mg/1 day) produced more effective suppression of salivary and plasma cortisol, with a superior performance compared to the standard high-dose (8 mg) DST.

Based on the parallelism of plasma cortisol and salivary cortisol lines and on their no coincident profiles, we identified that the criterion of 65% suppression of salivary cortisol corresponding to 50% suppression of plasma cortisol, after a high dose of DST, as consistent with CD. It is important to point out that Flack *et al.* (1992) showed that a greater degree of suppression (80%) of UFC should be used for the diagnosis of CD. Therefore, the present study also indicates the need for a higher degree of salivary cortisol suppression compared to plasma cortisol. In our opinion, these observations provide compelling evidence of the importance of using more stringent suppression criteria when interpreting the response of free cortisol (in urine or saliva) throughout dex suppression tests.

In addition, in the present study we measured salivary cortisol as an end point for standard tests such as DST and oCRH for the differential diagnosis of CS. High-dose DST is not required to discriminate between ACTH-dependent and ACTH-independent causes of CS and also it does not identify every patient with ACTH-dependent CS (Bruno *et al.*, 1985; Tyrrel *et al.*, 1986; Orth, 1995). In the absence of established criteria of salivary cortisol measurement as an end point parameter for the differential diagnosis of CS, we used 65% salivary cortisol suppression after 8-mg DST as a criterion for the diagnosis of pituitary disease. Using this more stringent criterion, salivary cortisol sensitivity seems to be higher than plasma cortisol sensitivity. However, due to the small number of patients with ectopic ACTH syndrome in the present series, further studies are still required to achieve the definitive criterion of suppression for salivary cortisol.

We also compared the diagnostic value of the salivary cortisol response to the oCRH test compared to plasma cortisol and ACTH. Once again, in the absence of criteria for the interpretation of the salivary cortisol response to oCRH, we evaluated this parameter using the established criteria for plasma cortisol (increment of 20%) and plasma ACTH (increment of 35%; Nieman *et al.*, 1993). Salivary cortisol after the oCRH test appeared to be as good as plasma cortisol or ACTH measurement in differentiating patients with CS. However, the oCRH test is also performed during IPSS (Oldfield *et al.*, 1991; Lienhardt *et al.*, 2001), and in this condition salivary cortisol is of no use.

It is important to point out that salivary cortisol has previously never been tested in the differential diagnosis of CS and these

data set the path for future analysis. In the absence of previous studies and the limited number of patients with ectopic ACTH syndrome in the present study, further studies are necessary to confirm these criteria for the interpretation of salivary cortisol in the differential diagnosis of CS. In conclusion, salivary cortisol, a free form of circulating cortisol, presents more profound suppression than plasma cortisol or ACTH in a dose–response pattern after different DSTs in patients with ACTH-secreting pituitary tumours. In addition, our data suggest that measurement of salivary cortisol might improve the sensitivity in DST as compared to plasma cortisol. Measurement of salivary cortisol is a simple, reliable and potential alternative tool during dynamic tests of pituitary–adrenal function for the differential diagnosis of CS.

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