glomerular lesions in the kidneys of hypokalemic and (or) diabetic patients (*19*).

By contrast to albumin, the urinary excretion of RBP seems to correlate to the metabolic control of Type II diabetes. Prospective trials should deal with a possible correlation between metabolic control, urinary RBP excretion, and micro/macrovascular complications in non-insulin-dependent diabetes. Another point of interest would be to test the validity of urinary RBP excretion as a predictor of mortality rates in Type II diabetes.

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Testosterone Concentration Is Increased in Whole Saliva, but Not in Ultrafiltrate, after Toothbrushing

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The concentration of testosterone in whole saliva is significantly increased (by 9%) after toothbrushing. In ultrafiltrates of saliva collected at the same time as the whole saliva, testosterone concentrations after toothbrushing were unchanged. In 88% of the 162 whole-saliva specimens, but not in the ultrafiltrates, we also measured higher hemoglobin concentrations after toothbrushing. We conclude that the increase of testosterone in whole saliva after toothbrushing can be attributed to a protein-bound fraction. For analytes that are bound to serum proteins, salivary measurements can give spurious results. This problem can be avoided by using as a diagnostic medium an ultrafiltrate of saliva collected directly in the mouth.

Indexing Terms: *saliva ultrafiltrate · free steroid · gingival exudate · hemoglobin · variation, source of*

Salivary testosterone has been used in many studies during the past decade for the diagnostic evaluation of androgenicity (for an overview, see *1, 2*). Circulating testosterone is substantially bound to three proteins: sex-hormone-binding globulin (SHBG)[1] and, with lower avidity, albumin and corticosteroid-binding globulin.

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[1] Nonstandard abbreviations: SHBG, sex-hormone-binding globulin; s_a, whole saliva after toothbrushing; s_b, whole saliva before toothbrushing; u_a, ultrafiltrate of saliva after toothbrushing; and u_b, ultrafiltrate of saliva before toothbrushing.

The bioavailable (i.e., androgenically active) fraction of testosterone is considered to be the portion that is present in saliva. However, there is an ongoing debate whether salivary testosterone better represents the free fraction of the hormone or the free plus the weakly bound fraction (see *1, 3*). Given this controversy, it is particularly important that salivary testosterone concentrations be accurately measured.

Because the salivary basal membrane excludes serum proteins, only 2–3% of the total testosterone concentration in serum is found in saliva. Therefore, the presence in saliva of serum or gingival exudate, which carries ~30-fold more testosterone per volume than does saliva, can lead to a false diagnostic indication. Indeed, it has been suggested that toothbrushing may lead to spuriously increased concentrations of salivary steroids (*4*), and a salivary SHBG concentration of only 0.1% of that in serum has been calculated to increase the measured testosterone concentration in saliva by 10% (*5*). No experimental data have yet been published to confirm these predictions.

Some commercially available assays claim to measure the "free" component of testosterone. It has been reported that SHBG interferes with reliable measurements of steroids in serum (*6, 7*). Because SHBG can also be present in whole saliva (*8*), caution is advised in the interpretation of measurements of salivary testosterone. Likewise, other salivary proteins can adversely affect the quality of steroid assays (*9*).

We have studied the effect of increased protein content in saliva, observed after toothbrushing, on the measured concentration of testosterone. We collected whole saliva before toothbrushing (s_b) and after toothbrushing (s_a), and an ultrafiltrate of saliva (*2*) before (u_b) and after (u_a) toothbrushing. The ultrafiltrate does not contain proteins, including SHBG. We found higher concentrations of testosterone after toothbrushing in whole saliva, but not in the ultrafiltrate.

Materials and Methods

Subjects and sample collection. Twenty-seven men (ages 20–40 years) with no apparent gum bleeding (self-reported) either before or after toothbrushing were selected for this study. Volunteers were instructed to brush their teeth normally, avoiding any increase in time or vigor. They collected whole saliva and ultrafiltrate of saliva immediately before toothbrushing. After toothbrushing, the subjects rinsed their mouths with water and immediately moved an osmotic device around in the mouth while they expectorated excess saliva (~3 mL) into a 10-mL polypropylene vial. The procedures followed were in accordance with the ethical standards defined in the Helsinki Declaration of 1975, as revised in 1983.

The ultrafiltrate (1 mL) was collected with an osmotic device kept in the mouth for 10 min as described before (*2*). The osmotic device consists of a small pouch made of semipermeable membrane that contains sucrose. The semipermeable membrane excludes molecules >12 000 Da. After collection, the pouch was stored in a polycarbonate container. All samples were stored at −20 °C until they were analyzed. Volunteers provided six sets of samples, each set collected at intervals >12 h.

Assays. We assayed testosterone by solid-phase radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) according to the manufacturer's instructions for salivary samples, with minor modifications. To measure the majority of the samples at between 20% and 80% of tracer binding on the dose–response curves, we increased the sample volume from the suggested 200 μL to 500 μL and adjusted all other reagents accordingly. The quality-control standards provided with the assay (low, medium, and high) yielded testosterone values within the expected ranges. This assay measures total testosterone concentrations with no apparent interference due to SHBG, according to the manufacturer's information.

We determined hemoglobin from erythrocytes in salivary samples with test strips (Chemstrip; Boehringer Mannheim Diagnostics, Indianapolis, IN) as described previously (*10*). We compared the readings before and after toothbrushing for each set of samples, using the semiquantitative scale of the test strips, and classified the hemoglobin content of the samples in two categories: (*a*) equal before and after toothbrushing and (*b*) higher after toothbrushing.

Statistical analysis. We performed a two-way analysis of variance (*11*), testing the hypothesis that testosterone concentrations are increased in whole saliva (s_a) but not in the ultrafiltrate (u_a) after toothbrushing. To eliminate variations of absolute testosterone concentrations among sample sets, we used the relative changes of testosterone concentrations for each sample set and performed two analyses with the same data. In Analysis 1, the two categories in the analysis of variance were as follows: A, the ratios s_a/s_b compared with ratios u_a/u_b; and B, comparison of the ratios among individuals. In Analysis 2, we analyzed the data by substituting s_b/u_b and s_a/u_a for the variances of the ratios in category A and B. In both analyses, we examined interaction between the variances A and B.

Results

Because the osmotic device excludes proteins, no ultrafiltrate sample (u_a and u_b) contained detectable amounts of hemoglobin. Of the 162 sets of whole saliva (s_b and s_a), 143 had greater concentrations of hemoglobin after toothbrushing than before; 19 sets showed no change; and in no whole-saliva sample did we find less hemoglobin after brushing than before. Excluding the samples without change in hemoglobin from the statistical analysis did not change the outcome of the results; therefore, we included results for all sample sets.

The average ratio of testosterone concentration of whole saliva after and before toothbrushing (s_a/s_b) was 9% higher than the average ratio of ultrafiltrate (u_a/u_b): 1.14 vs 1.05. This difference was significant ($F_{0.025}$) and confirms the proposed hypothesis. The average ratios for individuals before and after brushing, i.e., category B, were also significantly different ($F_{0.01}$); however, there was insufficient evidence for A × B interaction ($F_{0.05}$). In

other words, testosterone in ultrafiltrate does not generally increase in proportion to the increase in whole saliva caused by brushing.

After substituting the ratios s_a/u_a and s_b/u_b in category A, we obtained the same results: s_a/u_a, 1.16, was also 9% higher than s_b/u_b, 1.07 (significant, $F_{0.01}$); individual ratios (B) were significantly different ($F_{0.01}$), but they did not affect A (no interaction between A and B; $F_{0.05}$).

Discussion

These investigations showed that testosterone concentration in whole saliva increases significantly after toothbrushing. We attribute the 9% increase to sequestered hemoglobin and other accompanying blood proteins carrying bound testosterone. That the increase is from protein-bound testosterone after toothbrushing is supported by two observations: (*a*) hemoglobin increased in most of the subjects (88%), and (*b*) no increase of testosterone was observed in the protein-free ultrafiltrate.

It is not surprising that the increase in testosterone varies significantly among individuals. The stimulation of gingival and mucosal transudate that carries proteins into the mouth (*12*) depends on the intensity and technique of toothbrushing, the general status of the gum tissue of individuals, and even the type of toothbrush used. These factors will differ among individuals. An increase of protein concentration in saliva can also be attributed to microlesions in the oral mucosa, even in subjects without periodontal diseases (*13*). The increase of testosterone in whole saliva after toothbrushing does not seem to be correlated to the variation in increase among individual subjects (the interaction of variances was not significant at the 95% level).

We did not use the absolute values of testosterone for the statistical analysis because the high variation among individuals and among samples collected from single individuals on different days would have masked the changes. Instead, we compared the individual ratios s_a/s_b with the u_a/u_b ratios for each sample set, and also compared s_a/u_a with s_b/u_b, obtaining essentially the same result.

We used toothbrushing to stimulate mucosal and gingival transudation and, possibly, bleeding from microlesions. Much less prosaic activities may increase the protein content in saliva and subsequently cause misinterpretation of measured analytes. Passionate kissing has been shown to cause blood components to be detectable in whole saliva (*14, 15*). Total exclusion of proteins, e.g., by collecting an ultrafiltrate, seems to be the most rigorous and technically inexpensive method to ensure measurement of free analytes in saliva.

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