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Diurnal Changes in Salivary Amino Acid Concentrations

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Abstract. *It has been suggested that the features of saliva (e.g. fluidity, secretion and amino acid concentration) reflect physiological and psychological state of primates as well as subprimates, however, studies which revealed the relationship between the circadian rhythm and the concentrations of salivary amino acids have been limited. In order to better understand their physiological role, diurnal changes of salivary amino acids were investigated in three undergraduate students. Salivary amino acids were recovered after deproteinization with 5% trichloroacetic acid and determined by an amino acid analyzer. Most amino acids, except for methionine, cysteine and asparagine, were detected in the saliva. The intake of lunch or amino acid supplement transiently increased the salivary amino acids, and in the latter case, the amino acid levels returned to baseline within 10 minutes. Physical exercise also slightly elevated the salivary amino acid levels. During the university examination period, the secretion of saliva was slightly, but not significantly, increased, accompanied by the elevation of glycine, alanine, ornithine, histidine and threonine, and the decline of lysine, leucine, aspartic acid and hydroxyproline. Salivary amino acid levels may be useful to evaluate stressful conditions.*

Saliva contains various physiologically active substances and cells that maintain homeostasis. Several amino acids in the saliva may affect biological responses. Caries-free adults show elevated levels of lysine and arginine in the saliva, as compared with caries-susceptible adults (1), suggesting a cariostatic effect of these dibasic amino acids. A significant relationship has been reported between the concentration of ammonium (possibly derived from decomposition of urea or amino acids) and caries prevalence (2). Glycine stimulated the production of prostaglandin E_2 and cyclooxygenase-2 protein in interleukin-1β-stimulated human gingival fibroblast, suggesting its involvement in the pathogenesis of periodontitis (3). Supplementation of pigs with tryptophan in the diet reduced the basal plasma cortisol and noradrenaline concentrations, suggesting the possible anti-stress activity of tryptophan (4). The major volatile substance of tobacco smoke (acetaldehyde) easily dissolves into saliva during smoking. This acetaldehyde can be totally removed by a cysteine-containing tablet which is sucked during smoking. Cysteine can bind to acetaldehyde and eliminate its toxicity (5). Endogenous glutamate may alter hedonic response to suprathreshold umami substances (*e.g.* monosodoium glutamate) (*i.e.* the extent to which one feels pleasantness due to umami substance) (6). There are specific receptors for inhibitory amino acids [glycine and γ-aminobutyric acid (GABA)] and stimulatory amino acids (glutamic acid) in taste buds, and these amino acids exert their effects *via* their respective receptors (7-9). Changes in salivary composition correlate with disease susceptibility, disease state, or both. However, the use of saliva for diagnostic purposes is complicated by the gland-specific effects of circadian rhythm or diurnal variation, and therefore, studies of the circadian rhythm of saliva amino acid concentrations have been limited (10, 11). In order to better understand the role of salivary amino acids, changes in the concentrations of salivary amino acids in three undergraduate students were investigated.

Materials and Methods

Collection of saliva. Saliva was collected from three male undergraduate students (*), subjects A (21 years old), B (19 years old) and C (20 years old), at five or six time points in daily university life, according to the Guideline of the Intramural Ethics Committee (approved as No. A0902). The collection of saliva was carried out at 8:50, 10:40, 12:20, 13:00, 14:50, 16:40 and 20:00. Subjects A and B, but not subject C, took lunch. Subject C occasionally took an amino acid beverage at 8:00. The saliva sampling before and after amino acid beverage intake was carried out at 7:50, 8:00, 8:10, 8:20, 8:30

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Key Words: Diurnal change, saliva, amino acids, student life, stress.

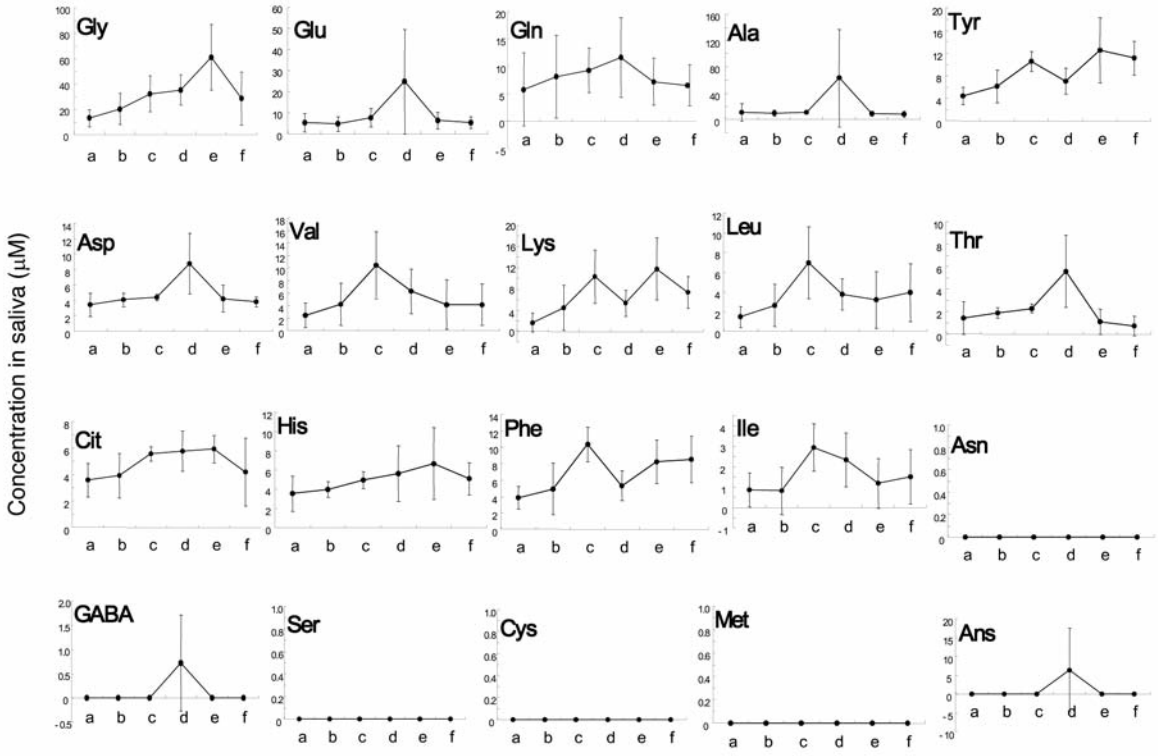

Figure 1. *Changes in amino acid concentration in saliva of Subject A. Saliva was collected at 8:50 (a), 10:35 (b), 12:20 (c), 13:00 (d), 14:50 (e) and 16:40 (f). Subject A ate lunch at 12:15-13:00. Each value represents the mean±S.D. of three assays.*

and 8:40, instead of the longer interval sampling schedule described above. Amino acids in the saliva were determined as described below.

Determination of free amino acids. Saliva (0.1 ml) was mixed with 0.1 ml of 10% trichloroacetic acid (TCA) (Wako Pure Chem Co., Tokyo, Japan). After centrifugation for 5 minutes at 21,000 ×*g* at 4˚C, the deproteinized supernatant was collected and stored at –30˚C. The supernatants (20 μl) were subjected to a JLC-500/V amino acid analyzer (JEOL, Tokyo, Japan) and amino acids were detected by the ninhydrin reaction (12).

Determination of secretion volume of saliva. The saliva was collected into a beaker for 5 minutes. The weight of corrected saliva was measured by chemical balance (Type 1702: Sartorius, Carl Zeiss, Tokyo, Japan), and divided by five to delineate the flow rate of saliva per minute. The pH of the saliva was measured by pH meter (F-8; Horiba Instruments Inc., Kyoto, Japan).

Statistical analysis. The mean values and standard deviations were calculated. The average values were compared by paired *t*-test. The value of statistical significance was set at the 0.05 level.

Results

Diurnal changes in amino acid concentrations. Subject A: The most abundant amino acid was glycine, followed by alanine, glutamic acid, valine, tyrosine, phenylalanine, serine, lysine, leucine, proline, glutamine, arginine, isoleucine, aspartic acid, and hydroxyproline. Histidine and threonine were present at much lower concentrations. GABA, asparagine, cysteine and methionine were below the detection limit (under 0.25 μM). After lunch time (12:20-12:40), most of the amino acid concentrations peaked rapidly, and then declined to basal level after or by 14:50.

Subject B: Subject B showed a similar temporal pattern in amino acid concentrations to subject A, except for GABA (Figure 2). Subject B had lunch, and exercised after school. The concentration of glycine, alanine, tyrosine, valine, lysine, leucine, histidine, phenylalanine, isoleucine and GABA in the saliva rapidly increased after lunch and exercise (playing volleyball after school). Cysteine, methionine and asparagine were below the detection limit.

Subject C: Subject C did not eat lunch, and therefore nearly constant levels of amino acids without any apparent peak were observed during the day time (8:50-18:00) (Figure 3). Cysteine, methionine and asparagine were below the detection limit.

Change in salivary amino acid levels after amino acid beverage intake. Subject C drank an amino acid beverage at 8:00, and the saliva was collected 10 minutes before and

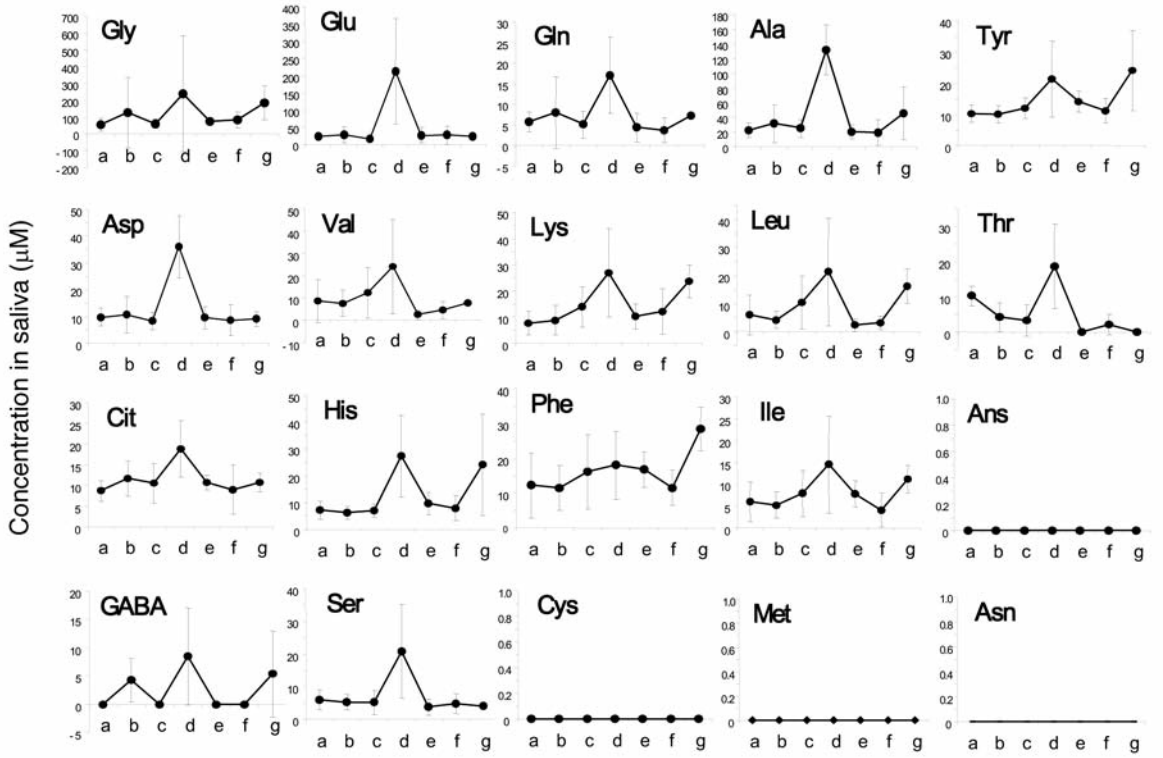

Figure 2. *Changes in amino acid concentration in saliva of Subject B. Saliva was collected at 8:50 (a), 10:35 (b), 12:20 (c), 13:00 (d), 14:50 (e), 16:40 (f) and 20.00 (g). Subject B ate lunch at 12:15-13:00 and played volleyball after school from 17:00 to 19:30. Each value represents the mean±S.D. of three assays.*

every 10 minutes after drinking it. By drinking the beverage, glycine, glutamic acid, glutamine, alanine, aspartic acid, valine, lysine, leucine, isoleucine, GABA and asparagine reached a maximum level within 10 minutes, and returned to their original levels within 10 minutes thereafter (Figure 4). On the other hand, tyrosine, threonine, citrulline, histidine, phenylalanine and serine did not show such drastic changes.

Effect of stress loading on salivary amino acid levels. Saliva was collected before and after a regular lecture and examination (both durations were 90 minutes), respectively, and amino acid concentrations, secretion and pH of saliva were measured. Each parameter showed a slight difference between samples taken before and after the lecture or examination, nevertheless these differences were not significant. Before the examination, subject C felt more stressful and fatigued, as compared with before the regular lecture (Table I). Under such stressful conditions, the secretion of saliva was slightly increased and the pH of saliva was maintained slightly lower than 7, however, these differences were not significant. The effect of stress on the amino acid concentration was significantly observed only for lysine reduction ($p<0.05$). Glycine, alanine, ornithine histidine and threonine were slightly increased, whereas glutamic acid, leucine, aspartic acid and hydroxyproline were slightly reduced. Asparagine, cysteine, methionine, GABA, serine and tryptophan were below the detection limit (data not shown).

Discussion

The present study demonstrated that (i) most amino acids, except GABA, methionine, cysteine and asparagine were detected in the saliva collected under the normal state, (ii) the intake of lunch or amino acid supplement resulted in the rapid increase and decrease of amino acids in the saliva which lasted less than 10 minutes; and (iii) physical exercise slightly elevated the salivary concentration of most amino acids. These data suggest that collection of saliva from normal subjects is not affected by the circadian rhythm if eating (food and drinks) and exercise were restricted. Moreover, since some salivary amino acid concentrations are significantly related to diseases (*e.g.* caries and periodontitis), the relationships between the level of those amino acids and the circadian rhythm remains to be investigated.

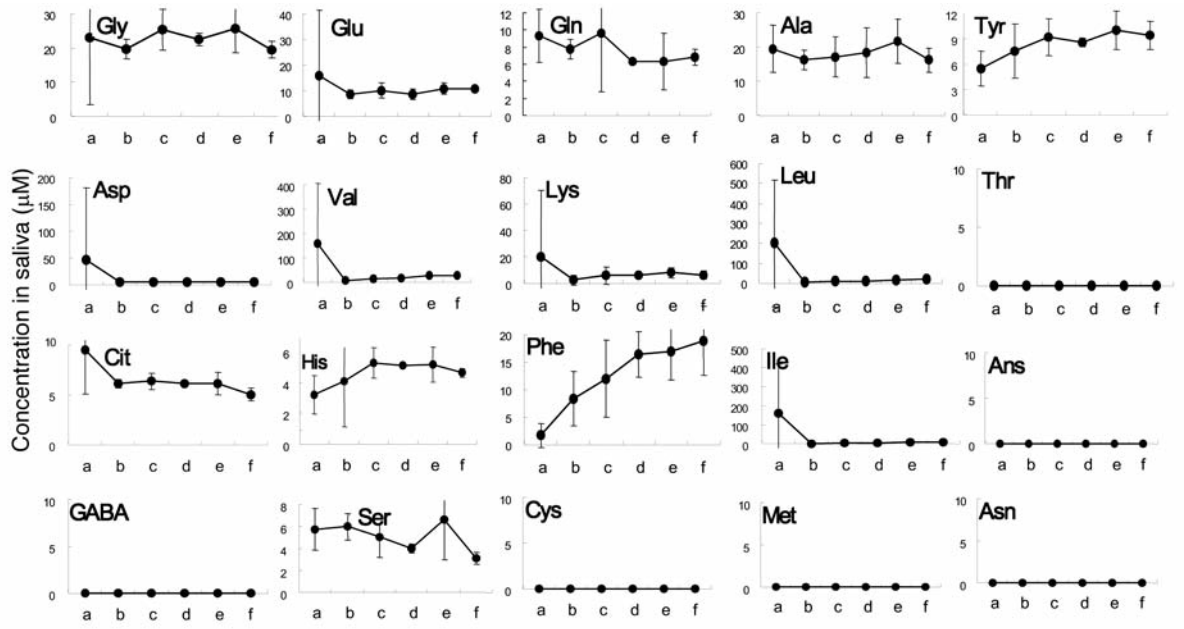

Figure 3. *Changes in amino acid concentration in saliva of Subject C. Saliva was collected at 8:50 (a), 10:35 (b), 12:20 (c), 14:50 (d) and 16:40 (e) and 18:00 (f). Each value represents the mean±S.D. of three assays.*

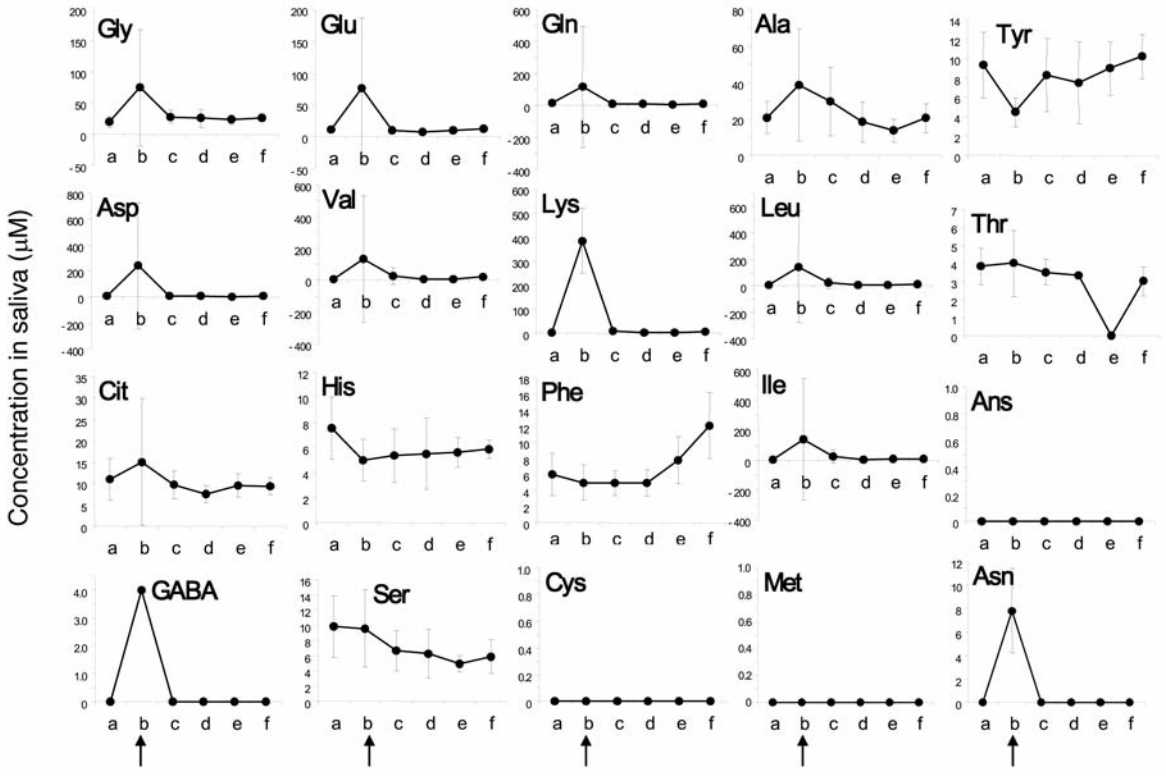

Figure 4. *Effect of intake of amino acid beverage on saliva amino acid levels. Subject C drank an amino acid beverage at 8:00 (arrow), and the saliva was collected every 10 minutes: at 7:50 (a), 8:00 (b), 8:10 (c), 8:20 (d), 8:30 (e) and 8:40 (f). Each value represents the mean±S.D. of three assays.*

Table I. *Effect of stress loading on the amino acid concentration, volume and pH of saliva of subject C.*

	Activity				
	Regular lecture		Examination		
	Before	After	Before	After	*p*-Value
Concentration (μM) in saliva					
Gly	39.2±9.3	31.0±14.7	41.0±18.0	46.7±18.1	0.43
Ala	17.4±4.9	14.0±3.1	20.7±6.4	19.2±5.6	0.19
Orn	13.4±7.5	11.5±6.3	14.0±10.6	18.5±8.6	0.51
Glu	13.6±2.4	13.3±6.0	12.6±6.5	11.6±6.9	0.70
Val	11.3±9.4	16.2±12.3	6.3±5.4	10.2±8.9	0.44
Tyr	13.7±3.6	12.0±5.8	11.8±6.2	12.2±3.8	0.79
Phe	10.8±3.3	12.0±6.5	7.1±6.4	10.3±3.8	0.45
Ser	10.8±1.3	6.8±2.4	15.1±10.0	10.7±4.8	0.18
Lys	9.8±5.2	9.2±4.1	2.2±3.8	3.7±6.3	0.03
Leu	8.1±4.1	11.3±7.6	4.2±3.6	6.7±5.8	0.33
Pro	11.4±9.9	6.1±5.3	6.0±2.6	9.5±10.5	0.87
Cit	8.1±0.94	8.1±1.4	9.2±2.1	6.8±4.1	0.95
Gln	8.4±2.7	6.9±0.36	6.6±2.1	4.8±4.5	0.13
Arg	6.5±1.6	5.2±1.7	7.5±2.3	5.9±1.4	0.09
Ile	5.4±5.0	9.0±3.5	2.3±3.4	6.0±5.2	0.38
Asp	2.6±3.6	1.5±2.1	<0.25	<0.25	0.19
Hypro	1.5±2.6	1.1±2.0	<0.25	<0.25	0.18
His	<0.25	<0.25	9.4±4.9	8.2±1.2	0.00
Thr	<0.25	<0.25	3.7±3.6	3.7±0.45	0.01
Flow rate of saliva (ml/min)	0.50±0.20	0.42±0.028	0.67±0.17	0.88±0.46	0.10
pH of saliva	6.89±0.39	6.68±0.22	6.56±0.75	7.04±0.26	0.96
Stress	None	Weak	Strong	Strong	
Fatigue	None	Weak	Strong	Strong	

Each value represents the mean±S.D. of three independent experiments. 'Stress' and 'Fatigue' were subjectively evaluated by Subject C.

Another factor that affects saliva composition is stress. It has been reported that salivary secretion declined during the student examination period (13). Salivary secretion was significantly lower in Parkinson's disease, and levodopa restored the salivary flow rate (14). The use of psychoactive drugs (antidepressant, antiepileptic, sedative, antipsychotic, hypnotic or sedative-hypnotic drugs) significantly reduced the salivary flow rate (15). On the other hand, the mental stress applied by computer tasks increased both the secretion and cortisol concentration of saliva, especially in aged women (16). We found that salivary secretion of subject C was slightly elevated during the examination period, in disagreement with a previous report (13). This discrepancy may be due to the difference of sensitivity of each student to an examination. There was a possibility that tryptophan, which has been reported to be involved in mood disorder, dementia and stress (17-19), may have affected the present result. However, this possibility seems to be low, since tryptophan was not detected in the saliva of any of the subjects (data not shown). We found that concentrations of glycine (an inhibitory amino acid), alanine, threonine and histidine in the saliva were slightly elevated, whereas those of glutamic acid (an excitatory amino acid) and lysine were reduced. This enhanced ratio of glycine/glutamic acid may reflect the change from the excited state to the depressed state during the examination. GABA, another inhibitory amino acid, was below the detection limit.

It has been reported that fewer than 11% of most salivary amino acids, except for alanine and proline, are L-enantiomers (20). We therefore have analyzed only the changes in the concentrations of L-enantiomers, but not D-enantiomers of each amino acid. The possible changes in D-alanine and D-aspartate derived from submandibular gland and epithelial cells (20) before and after stress remain to be investigated.

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