

Biochemical and Clinical Factors Influencing Oral Malodor in Periodontal Patients

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THE AMOUNTS OF VOLATILE SULFUR COMPOUNDS (VSC) and methyl mercaptan/hydrogen sulfide ratio in mouth air from patients with periodontal involvement were 8 times greater than those of control subjects. Our studies demonstrated that, in patients with periodontal disease: 1) the concentration of disulfide, which is converted to VSC, increased in proportion to the total pocket depth; 2) 60% of the VSC was produced from the tongue surface; 3) the amount of tongue coating was 4 times greater than in control subjects; and 4) VSC production and the methyl mercaptan/hydrogen sulfide ratio of the tongue coating were increased. 2-Ketobutyrate, which is a byproduct of the metabolism of methionine to methyl mercaptan, was higher in the saliva of patients with periodontal disease. This implies that metabolism of methionine to methyl mercaptan increases in the oral cavity of patients with periodontal pockets. Since free L-methionine, rather than protein, is the main source for methyl mercaptan, we estimated the methionine supply from the gingival fluid into the oral cavity of patients with periodontal involvement. The results showed that the ratio of methionine to whole free amino acids was significantly higher than that of cysteine. Our studies suggest that not only microorganisms, but also the tongue coating and gingival fluid are factors which enhance VSC production in patients with periodontal disease. *J Periodontol* 1992; 63:783-789.

Key Words: Periodontal pockets; saliva/analysis; methyl mercaptan; hydrogen sulfide; tongue, coated; 2-ketobutyrate/analysis; methionine/analysis; gingival fluid/analysis; sulfides, volatile/analysis.

Periodontal disease frequently involves pathological oral malodor, which is caused mainly by volatile sulfur compounds (VSC), such as hydrogen sulfide, methyl mercaptan, and dimethyl sulfide.^{1,2}

From ancient times,^{3,4} people have known that periodontal disease causes halitosis. Hippocrates described a remedy for pathological oral malodor³ and said, "If the gingiva become healthy again, the offensive odor vanishes." Some scientific studies^{5,6-8} conducted during the past 50 years have shown that periodontal disease causes the offensive odor. Recently, Tonzetich et al.⁹⁻¹⁵ provided evidence that pathological malodor may accelerate periodontal disease. VSC increase the permeability of the oral mucosa and collagen solubility^{9,11} and decrease protein or collagen synthesis^{10,15} and thus may be considered to be involved in the pathogenesis of periodontal disease. Therefore, it is important to comprehend the factors which influence pathological oral malodor in order to conduct research into the

pathogenesis of periodontal disease and develop treatments for pathological oral malodor.

Microbiological studies^{16,17} have demonstrated that periodontal pathogenic microorganisms contribute to increased VSC production, in particular that of methyl mercaptan, in the oral cavity. Therefore, it has been suggested that *Fusobacterium*, *Porphyromonas gingivalis*, and other microorganisms have important roles in the pathogenesis of halitosis.^{16,17} In order to gain understanding of the pathology of halitosis associated with periodontal disease, we have conducted studies to determine clinical and biochemical factors which accelerate the production of VSC.

Association Between Halitosis and Periodontal Status

Elevated concentrations of VSC occur frequently in mouth air from patients with periodontal disease.^{8,18} Tonzetich⁸ demonstrated that the VSC concentration in mouth air increased with the total pocket depth, and we found, using the gas chromatography method he developed,² that this was higher in patients with probing depths of 4 mm or more than in subjects with probing depths of less than 4 mm (Fig. 1).¹⁸ In particular, the methyl mercaptan concentra-

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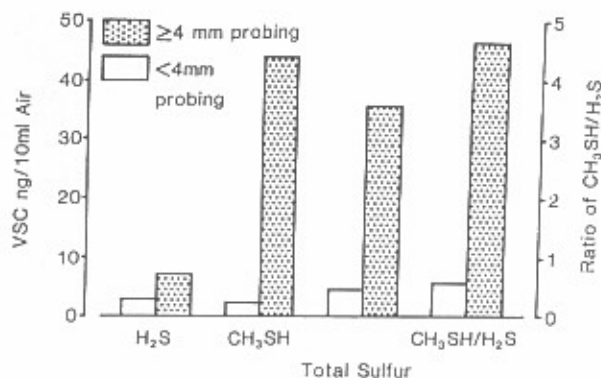


Figure 1. VSC production in mouth air from subjects ($n = 17$) with a gingival probing depth of 4 mm or more. In comparison with subjects ($n = 14$) with a probing depth of less than 4 mm, a large amount of VSC production was observed, and the methyl mercaptan/hydrogen sulfide ratio was markedly increased.

tion was significantly higher in patients with periodontal disease than in controls (44.0 ng/10 ml vs. 2.6 ng/10 ml, $P < 0.01$) (Fig. 1).

The bleeding index, which is derived from the number of bleeding points caused by probings, was used to compare the extent or severity of periodontitis. Figure 2 shows that the total sulfur content in 10 ml of mouth air increased in proportion to the bleeding index.⁸ The production of hydrogen sulfide in periodontal pockets was demonstrated by inserting leaded filter papers into the pockets; it must be noted that this method is not quantitative.⁷ Furthermore, Coil and Tonzetich¹⁹ have indicated increased VSC production in periodontal pockets by employing a special device to collect the gases.

It is evident that periodontal disease causes high concentrations of VSC in mouth air, with consequent quantitative changes of bad breath, although there is little information available about the qualitative change of bad breath.¹⁸

Since the oral environment of patients with periodontal disease may differ from healthy subjects, we wondered whether the composition of VSC associated with pathological odor was the same as that of physiological oral malodor. We determined the methyl mercaptan/hydrogen sulfide ratio by gas chromatography method.¹⁸ These two compounds, rather than dimethyl sulfide, have been considered to be the main components of VSC in mouth air.^{2,20} We found that the methyl mercaptan/hydrogen sulfide ratio in patients with probing depths of 4 mm or more was much higher than that of controls (4.64 vs. 0.58, $P < 0.01$) (Fig. 1),¹⁸ and that it increased in proportion to the bleeding index which reflects the extent of periodontal disease (Fig. 2).¹⁸

The maximum probing depth was determined and compared with the methyl mercaptan/hydrogen sulfide ratio in each patient with periodontal disease. Figure 3 shows that the ratio increased with probing depth.¹⁸ The group with probing depths of 3 mm or less had a ratio of 0.37 ± 0.10 (mean \pm SE, $n = 9$); with 4 mm probing depths the ratio

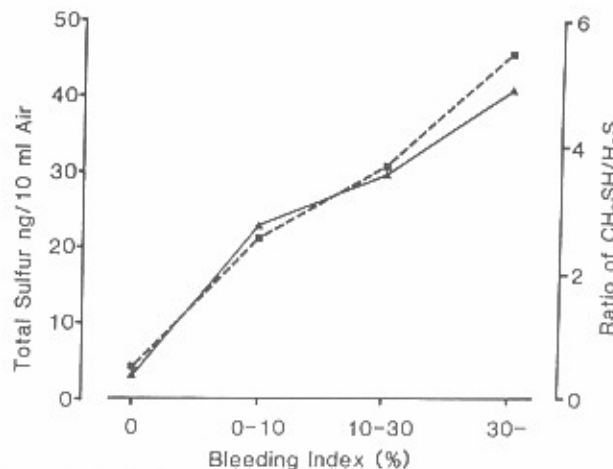


Figure 2. Total sulfur concentration and ratio of methyl mercaptan/hydrogen sulfide in relation to bleeding index. VSC production and the ratio increased with bleeding index. Each value is an average of each class, the numbers in each class being as follows: 0; $n = 11$, 0-10; $n = 7$, 10-30; $n = 4$, 30-; $n = 9$. Broken line: ratio of methyl mercaptan. Solid line: total sulfur.

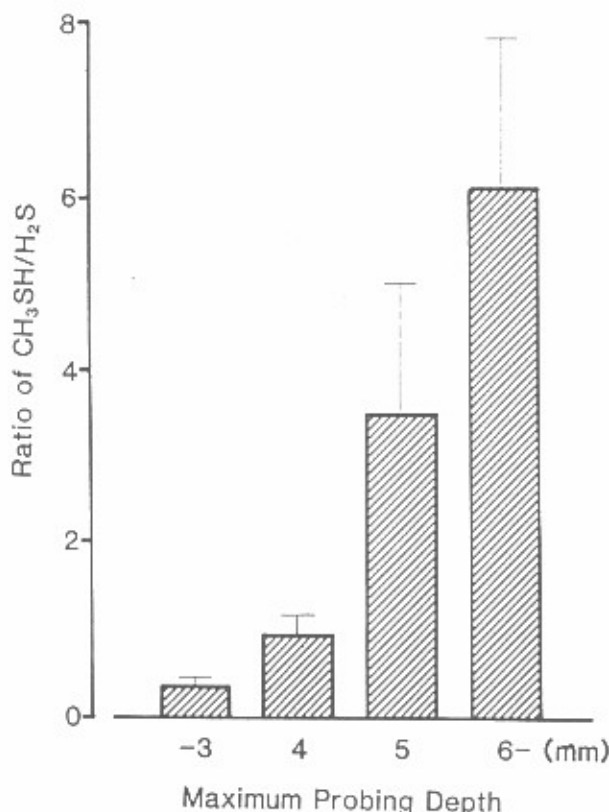


Figure 3. Ratio of methyl mercaptan/hydrogen sulfide and maximum probing depth in each subject. The ratio increases with depth. The numbers in each class are as follows, 0-3 mm, $n = 9$; 4 mm, $n = 6$; 5 mm, $n = 8$; 6 mm-, $n = 11$.

was 0.96 ± 0.23 (mean \pm SE, $n = 6$); it was 3.49 ± 1.48 (mean \pm SE, $n = 8$) with 5 mm depths; and 6.10 ± 1.71 (mean \pm SE, $n = 11$) with depths in excess of 5 mm. Therefore, these results indicate that methyl mercaptan

is the main component of VSC in patients with periodontal involvement, hydrogen sulfide is the main constituent in orally healthy subjects, and the methyl mercaptan/hydrogen sulfide ratio increases with the periodontal disease severity. It can be implied from these results that estimating the amount of hydrogen sulfide, rather than methyl mercaptan, would be appropriate for evaluation of physiological malodor, especially in patients with strong psychosomatic tendencies because usually it appears to be only hydrogen sulfide that causes the objectionable smell in such patients.²¹ Recently, portable bad breath detectors¹⁹ have been introduced into the clinical field.²¹⁻²³ Since all of these cannot distinguish between hydrogen sulfide and methyl mercaptan, they are not applicable to our purpose. Although some portable detectors are less reliable than gas chromatography, one^{19,21,23} may be useful for the screening of malodor in clinics.

We found that the methyl mercaptan concentration in mouth air from patients with periodontal disease increased compared with normal subjects. It is believed that hydrogen sulfide is produced from thiols, such as cysteine,^{24,25} and methyl mercaptan originates from methionine.¹⁷ However, it has been suggested that hydrogen sulfide can be converted to methyl mercaptan in the oral cavity.²⁶ We investigated whether methionine metabolism, outlined below,¹⁷ is enhanced in the oral cavity of patients with periodontitis.



2-Ketobutyrate is a byproduct of the methyl mercaptan production process, so we estimated its concentration in saliva with the high performance liquid chromatography (HPLC) procedure we have developed. The saliva samples were obtained from the mandibular mucobuccal fold. The protein was removed by freezing, and then adding perchloric acid to a final concentration of 5%. The 10,000 × g supernatant was then degassed by aspiration for 5 minutes. Water was added to adjust the original sample volume, 25 µl samples were added with 50 µl 1 M acetate buffer (pH 5.0), and the sample was derivatized with 20 µl 0.1% w/v 3-methyl-2-benzothiazoline hydrazone at room temperature for 30 minutes. The HPLC conditions used were as follows: a Pico tag column⁸ was eluted at 38°C with buffer A, which consisted of 0.05% triethyl amine, and 5.0% acetonitrile buffered at pH 5.5. The column was eluted at a flow rate of 0.5 ml/min for 4 minutes; after which the Pico tag column was eluted with buffer B (60.0% acetonitrile). The acetonitrile was increased to 100% for 30 seconds, and peaks were detected and recorded at an absorbance wave length of 254 nm. The results demonstrated that 2-ketobutyrate increased dramatically in the mixed saliva from patients with periodontal disease compared with normal

Table 1. 2-Ketobutyrate in Saliva

	Periodontitis (n = 38)	Health (n = 16)	
2-Ketobutyrate (nmole/ml)	32.7 ± 4.1	0.6 ± 0.5	P < 0.001*
Total pocket depth (mm)	202.7 ± 23.6	—	

*Unpaired *t*-test.

DISULFIDE DISTRIBUTION IN SALIVARY CELLULAR ELEMENTS

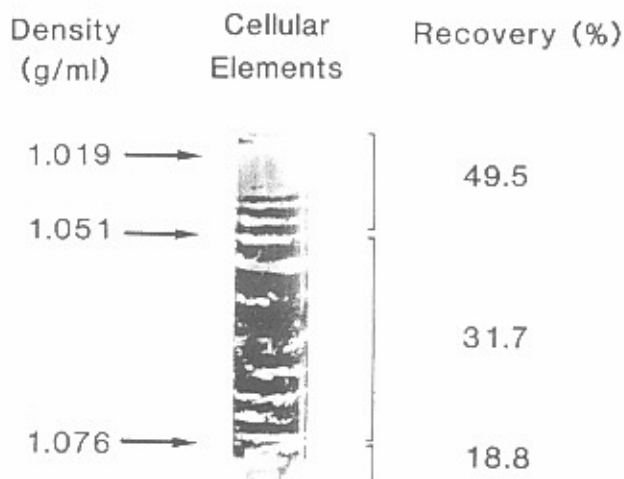


Figure 4. Distribution of disulfide in salivary cellular elements. Cellular elements were fractionated by percoll gradient centrifugation. Almost 50% of disulfide was distributed in the lower-density fraction (less than 1.051 g/ml), consisting of relatively intact cells, in comparison with other fractions.

subjects (Table 1), and support strongly the observations that the production of methyl mercaptan from methionine is accelerated in patients with periodontal disease.

Saliva

It has been suggested that saliva is one of the main sources of bad breath, because VSC are produced from proteins and sulfur-containing amino acids present in saliva.^{1,20,24,27}

Since filtration removes 90% of thiols and disulfide from whole saliva, though dialysis does not affect the thiol and disulfide content in the saliva, and sonication of whole saliva increases these concentrations in the supernatant, Ton-zetich and Johnson²⁴ concluded that the cellular elements of saliva are the principal sources of the thiol and disulfide groups utilized for the production of hydrogen sulfide.

We analyzed the distribution of thiols and disulfide in salivary cellular elements with a percoll density gradient centrifugation technique²⁸ and found that disulfide is distributed mainly in the intact cells (Fig. 4), whereas the thiol concentration was lower in intact than damaged cells. A saliva putrefaction study indicated that cell destruction increased the specific gravity of the cells and decreased disulfide content.^{28,29} In light of these results, we postulated

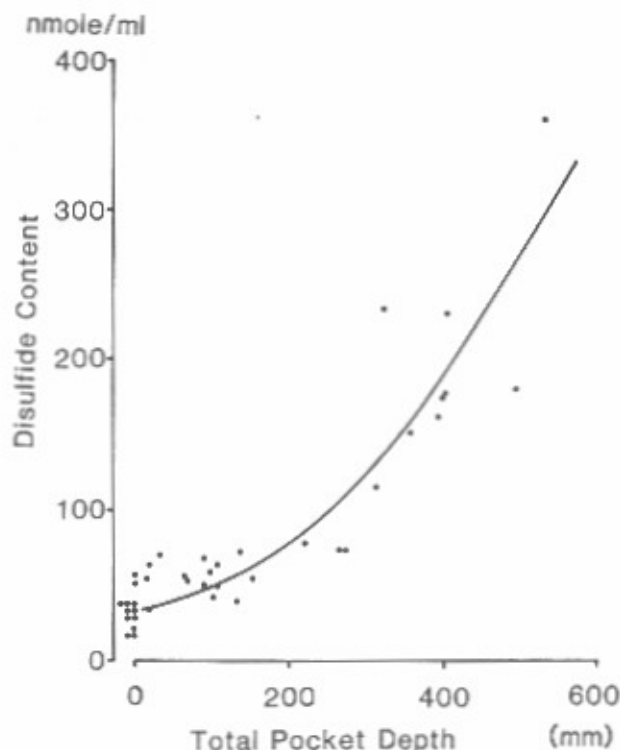


Figure 5. Disulfide in saliva and periodontal disease. Disulfide content (nmol/ml) in saliva was increased with total pocket depth in each patient. A regression curve was obtained, $Y = 40.5275 + 0.0299X + 0.0009X^2$, with a coefficient of determination of 0.8407 ($P < 0.01$).

that the contents of the intact cells would be very rich sources of VSC.

The cellular elements in saliva comprise epithelial cells released from the oral mucosa, microorganisms, and leukocytes.³⁰ The epithelial cells contain keratin, cysteine-rich protein, although keratinization is not complete. Leukocytes also possess large quantities of sulfur-containing amino acids, which could be utilized for VSC production.³⁰ Since leukocytes migrate from periodontal pockets, their number in saliva increased in patients with periodontal disease, although epithelial cells did not increase significantly.³⁰ Furthermore the number of leukocytes correlated with some periodontal evaluations³⁰ and methyl mercaptan concentrations in mouth air. It has thus been suggested that leukocytes may increase the source of VSC in saliva of patients with periodontitis.

Our study indicated that the salivary thiol concentration is not increased significantly in patients with periodontal disease.²⁵ Thiol is probably too reactive to exist in its free form for long, therefore, no significant difference between the thiol concentrations of control and periodontitis groups would be found. However, disulfide is more stable than thiol, and we found that the disulfide content of saliva correlated with the severity of periodontal disease (Fig. 5).²⁵ A regression curve was obtained ($Y = 40.5275 + 0.0299X + 0.0009X^2$, with a correlation coefficient of 0.8404, $P < 0.01$). The disulfide content correlated also with the pocket

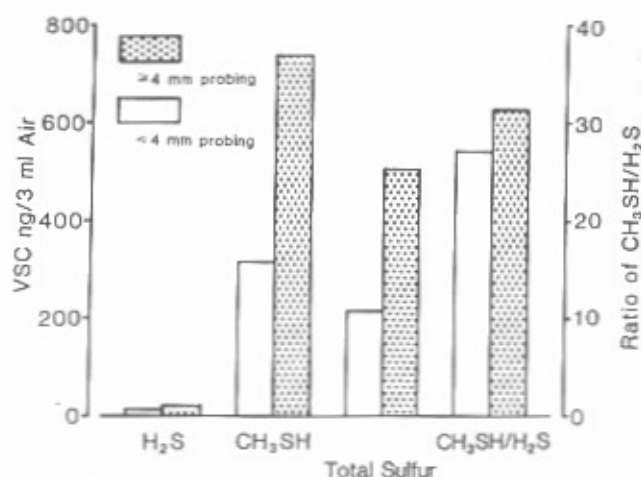


Figure 6. VSC production in a saliva putrefaction system. VSC production in saliva from subjects ($n = 17$) with a probing depth of 4 mm or more is almost double that of the control, but the ratio of methyl mercaptan is the same as the control average.

share ratio and mean pocket depth.²⁵ Therefore, on the basis of these results, the salivary disulfide concentration appears to reflect the periodontal status. There is a plausible explanation for such an increase of disulfide: an increase of the salivary protein content may increase the available disulfide in saliva, as the sulfur-containing amino acids of protein molecules comprise disulfides. However, our results demonstrated that the disulfide concentration (/mg protein) also increased in proportion to the severity of periodontal disease (data not shown). Therefore, we speculated that the content of sulfur-rich proteins or sulfur-containing amino acids increased in saliva from patients with periodontal involvement.

Our study demonstrated that VSC concentrations and the methyl mercaptan/hydrogen sulfide ratio increased in mouth air in proportion to the bleeding index. As the bleeding index value and concentration of blood are mutually related, it has been suggested that other blood components, as well as leukocytes, originating from periodontal pockets may participate in acceleration of VSC production. Gibbons and MacDonald³² found that most strains of *Porphyromonas gingivalis*, including some periodontally pathogenic strains, required hemin for their growth, and the growth rate increased in proportion to the hemin concentration. Therefore, hemin originating from blood may enhance the bacterial activity, which produces methyl mercaptan in saliva or periodontal pockets.

A saliva putrefaction system has been employed¹⁸ to estimate whether saliva from patients with periodontitis contributes to accelerated VSC production. The head-space air in the Teflon-coated glass tube (17.5 ml volume) was replaced with nitrogen gas, 1 ml saliva was incubated for 24 hours at 37°C, and 3 ml head-space air was analyzed by gas chromatography. Figure 6 shows that the amount of VSC in head-space air from incubated saliva of patients with periodontal disease increased compared with normal subjects. However, this increase is very slight compared

with that in mouth air (Figs. 1 and 6). The methyl mercaptan/hydrogen sulfide ratio in saliva from patients with periodontitis was not different from the ratio in control, although the ratio increased dramatically in mouth air from patients with periodontal disease. There is no doubt that the elevated putrefaction activity of saliva contributes to increased VSC production in periodontal disease, but the contribution of saliva to the increased methyl mercaptan/hydrogen sulfide ratio was less than we had expected. Therefore it has been strongly speculated that some factors other than saliva may influence methyl mercaptan production in mouth air from periodontal patients.

Tongue Coating

Kaizu³³ had suggested that the tongue coating may not play an important role in the production of VSC in patients with periodontal disease, whereas removal of the tongue coating did reduce VSC in orally healthy subjects.^{8,33} It has been reported that tongue coating removal does not prolong suppression of methyl mercaptan production in patients with periodontal disease.³³ However, these studies combined oral rinsing or tooth brushing with tongue coating removal, and, therefore, the amount of VSC produced by the tongue coating was not estimated accurately.

A preliminary study demonstrated that the tongue coating volume tended to increase in cases with periodontal involvement.³⁴ The tongue coating comprises epithelial cells released from the oral mucosa, microorganisms, and leukocytes from periodontal pockets.^{34,35} As these cells, in particular leukocytes, are increased in saliva from patients with periodontal disease and accumulate on the tongue surface, tongue coating would be expected to be increased in patients with periodontal disease. Thus we have postulated that the tongue coating may be an important factor in pathological and physiological oral malodor, contrary to this previous study. Therefore, the following experiments were performed to determine the effect of tongue coating on VSC production in patients with periodontal disease.¹⁸

The subjects were instructed to abstain from oral hygiene, including oral rinsing and ingestion of food and liquid, on the morning of the test. After VSC analysis, cotton rolls were put around the tongue to exclude moisture and saliva; saliva on the dorsal surface of the tongue was removed with a stream of air and pure pulp tissue paper;⁴ the tongue coating from the terminal sulcus to the apex of the tongue was removed carefully with a tongue scraper of the small spoon type to avoid contaminating the oral cavity and saliva with coating material; and the dorsal surface was cleaned with cotton pellets immersed in physiological saline. The VSC analyses of mouth air were repeated after removal of the tongue coating. Unlike previous studies of tongue coating,^{8,33-35} which have usually employed classification by inspection to estimate the volume of tongue coating on the tongue surface, we measured the wet weight

Table 2. VSC Production from Tongue Coating

	Wet Weight (mg)	VSC (ng/10ml)	CH ₃ SH/H ₂ S Ratio
Control (n = 6)	14.6	4.3 (8.3*)	1.0
Periodontitis (n = 17)	90.1	18.6 (36.5*)	31.3

*Total VSC concentration in mouth air before tongue coating removal.

of the tongue coating. The VSC production by the tongue coating was calculated by subtracting the amount of VSC produced after tongue cleaning from the initial amount prior to cleaning. Table 2 shows that the group with periodontal disease (> 4 mm probing depth) had far more tongue coating than the controls (14.6 mg vs. 90.1 mg, $P < 0.01$), although the salivary flow rates of the two groups did not differ, and VSC production by the tongue coating of periodontal disease patients was estimated to be more than 4 times that of the controls (< 4 mm probing depth). Furthermore, the methyl mercaptan/hydrogen sulfide ratio was much higher in patients with periodontal disease than in controls (31.3 vs. 1.0, $P < 0.01$). It also has been demonstrated that much more VSC, especially methyl mercaptan, was produced on the tongue dorsal surface in patients with periodontal disease than in orally healthy subjects, and immediately after removal of the tongue coating the amount of VSC was reduced by almost half the initial amount. Therefore, the tongue coating must be an important factor which accelerates VSC production in patients with periodontal disease as well as in orally healthy subjects.

As the VSC composition produced by the tongue coating changed dramatically in patients with periodontal disease, these results indicate that the composition of precursors of VSC or bacterial characteristics of the tongue surface may differ in patients with periodontal disease from those in orally healthy subjects.

Gingival Fluid

Methyl mercaptan production from methionine is accelerated in mouth air from patients with periodontal disease. Tonzetich and McBride⁶ demonstrated that a pathogenic strain of *Porphyromonas* produced 12 times as much methyl mercaptan as hydrogen sulfide. This study implies that periodontal pathogenic microorganisms do accelerate production of methyl mercaptan in mouth air.

Moreover, there is little information in the literature concerning the relationship between precursors of VSC and periodontal disease.²⁵ We found that the salivary disulfide content increased in patients with periodontal disease, but as disulfide originates from cysteine or cystine, the disulfide content would have no effect on methyl mercaptan production.

The VSC concentrations in mouth air at 2 minutes after mouth rinsing with mouthwashes, such as L-cysteine (2 mM), L-methionine (2 mM), and 0.5% casein have been

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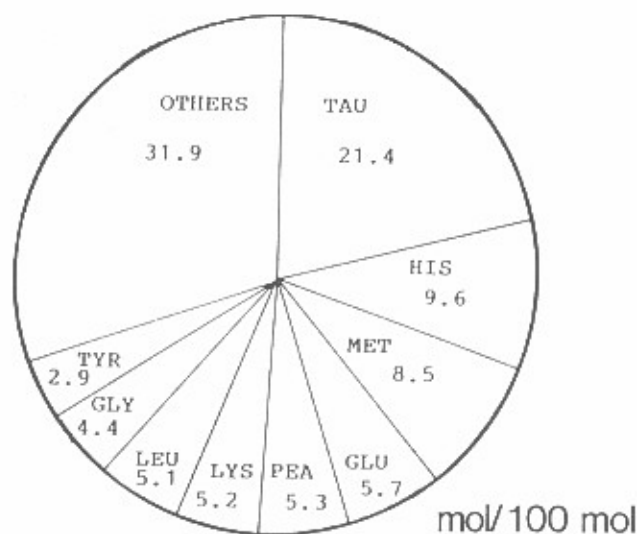


Figure 7. Amino acid composition of gingival fluids from patients with periodontal disease. Taurine (TAU) ratio is markedly high in comparison with others ($n = 23$).

determined.³⁶ It has been demonstrated that only free methionine is a good source of methyl mercaptan *in vivo*, so we investigated the free methionine supply for methyl mercaptan production. The concentration of methyl mercaptan in mouth air correlated with the severity of periodontal disease, which increased the gingival crevicular fluid flow rate. Therefore, it has been speculated that gingival crevicular fluid may be a continual supply of free methionine and other VSC precursors in patients with periodontal involvement.

The free amino acid composition of gingival crevicular fluid from the periodontal pocket was determined using a Wakosil-PTC column** with gradient system, which we developed to separate standard AN-type amino acids from physiological free amino acids. Samples were collected from the upper incisors pockets with filter strips, the papers were immersed in 100 μ l ice-cold water, and sonicated. The protein was removed by Sep-pack C18[†] and samples were derivatized. We found that 99% of methionine and cysteine were recovered in this system. The results showed that taurine was present in the greatest amounts among the free amino acids in gingival fluid (Fig. 7). There are many metabolic pathways of cysteine, but two are believed to predominate. One produces pyruvic acid, ammonium ions, and hydrogen sulfide, and the other taurine. The latter pathway may be more active in the periodontal pockets or tissues.

The ratio of methionine to whole free amino acids in gingival fluid from periodontal pockets was significantly higher than those of cysteine and cystine (Fig. 8). This suggests that gingival fluid is a good source for methyl mercaptan rather than hydrogen sulfide production.

We concluded that the periodontal pocket possesses ap-

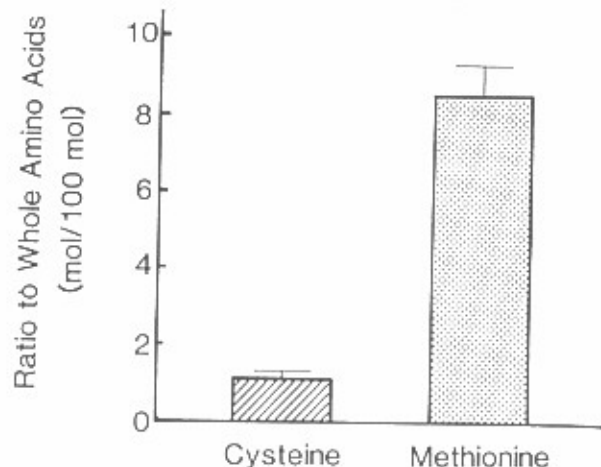


Figure 8. Methionine and cysteine ratios in gingival fluids from patients with periodontal disease. Methionine concentration is overwhelmingly higher than that of cysteine. Cystine value expressed as cysteine equivalent is also included.

propriate conditions for methyl mercaptan production, such as periodontal pathogenic microorganisms and an adequate supply of free methionine. Coil and Tonzetich¹⁹ reported that very high concentrations of dimethyl sulfide, to which methyl mercaptan was dimerized, occurred in periodontal pockets. This supports the finding that methionine metabolism is accelerated in periodontal pockets by the factors mentioned above. The elevated methyl mercaptan production in periodontal pockets then would accelerate periodontal disease, since methyl mercaptan has been demonstrated to be more cytotoxic than hydrogen sulfide.^{11,37}

Conclusion

Production of VSC in patients with periodontal disease occurs in periodontal pockets and on the tongue surface. However, saliva may make only a minor contribution to pathological odor production. Methyl mercaptan, rather than hydrogen sulfide, is the main component of pathological malodor, whereas the converse applies in orally healthy subjects. The salivary disulfide concentration correlates with the severity of periodontitis, but may not contribute to the increased methyl mercaptan concentrations. The levels of 2-ketobutyrate are increased in the oral cavity of patients with periodontal disease, which indicates that the metabolism of methionine to methyl mercaptan is accelerated. The supply of methionine from gingival fluid is greater than that of cysteine or cystine.

Acknowledgments

We thank Drs. A. Hasegawa, S. Hamaguchi, and A. Sakai, Department of Periodontics, The Nippon Dental University, School of Dentistry at Niigata, for their cooperation; we appreciate greatly the assistance of Dr. M. Rosenberg, Tel Aviv University; and we are very grateful to Dr. J. Tonzetich, The University of British Columbia, for his excellent discussion and suggestions to us.

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