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Salivary Myeloperoxidase of Young Adult Humans[1] (41822)

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Abstract. Leukocytes, principally polymorphonuclear leukocytes (PMNs), enter the oral cavity where they release a portion of their constituents, including myeloperoxidase, into oral fluids. A greater number of PMNs in the oral cavity are associated with oral inflammation. However, the quantitative contribution of the PMN to oral fluids, including saliva, during various conditions is poorly understood. An assay method based on the adsorbance loss at 278 nm from the reaction of the myeloperoxidase product hypochlorous acid with monochlorodimedon to yield dichlorodimedon was developed for the quantitation of salivary myeloperoxidase. Myeloperoxidase was determined in supernatants of whole saliva obtained at low and moderate flow rates and in parotid saliva collected during moderate and pronounced stimulation from young adults with minimal oral inflammation. The greatest myeloperoxidase activity was in whole saliva supernatants collected at low flow rates where PMN products have an opportunity to accumulate. Lesser quantities of myeloperoxidase were found in both the whole saliva supernatants and parotid saliva obtained at the faster flow rates. Low flow rate whole saliva supernatants contained about 25% of the myeloperoxidase in the PMNs which enter the oral cavity. Myeloperoxidase is responsible for a significant portion (15–20%) of the total peroxidase activity in supernatants of whole saliva obtained at low flow rates. Preliminary results indicate that young adults with phenytoin-associated gingival overgrowth or who smoke have more myeloperoxidase activity in low flow rate whole saliva.

Human whole (mixed) saliva contains a complex peroxidase system. Major components of this system include different forms of lactoperoxidase secreted by the salivary glands and myeloperoxidase from polymorphonuclear leukocytes (PMNs) which enter the oral cavity (1–3). The peroxidases, because of their likely participation in the oral antimicrobial system, have been the subject of major research interest. This interest has included possible relationships among oral fluid peroxidase activity, the quantity of peroxidase reactants and metabolites, and clinical conditions. Whole saliva and gingival fluid from subjects with gingival inflammation contain greater amounts of peroxidase (4–6). Increased whole saliva peroxidase has been reported to occur at the time of (7) and prior to (8) ovulation. Individuals with phenytoin-associated gingival overgrowth have more peroxidase in supernatants of whole saliva collected at low flow rates (9). The source of the additional peroxidase was not identified in the above studies. However, it was pointed out by some of the authors that myeloperoxidase from PMNs could account for at least a portion of the increased enzyme activity (5, 6, 9). Large numbers of leukocytes, principally PMNs, in the gingival crevice are a feature of both acute and chronic forms of gingivitis (10–12). Greater numbers of PMNs are found in the oral cavity in association with oral inflammation by an oral rinse technique (13–16). PMNs release some of their contents upon ingestion of bacteria or during contact with plaque (17–20). Furthermore, PMNs rapidly lyse when they enter saliva (13).

Quantitation of myeloperoxidase would aid in identifying the source of the additional salivary peroxidase activity in clinical conditions such as those mentioned above. Previous methods used for the quantitation of whole saliva peroxidase measured the total activity from various peroxidases and, thus, did not permit estimation of the contribution from different sources. Myeloperoxidase from PMNs, in contrast to peroxidases from most other sources, uses chloride as a cofactor to generate hypochlorous acid (21). Monocytes also contain myeloperoxidase and a peroxidase is present in eosinophils which can oxidize

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Cl^- at low pH (22). These later two cells, however, seldom are present in the oral cavity in numbers sufficient for a significant contribution to Cl^- oxidation. Chloroperoxidase is quantitated based on its ability to produce hypochlorous acid (23). The aims of the research described in this paper were, first, to adapt the chloroperoxidase procedure to the analysis of myeloperoxidase, and, secondly to determine the presence of and the amount of myeloperoxidase in supernatants of whole saliva collected at low and moderate flow rates and in parotid saliva obtained during moderate and pronounced stimulation from young adults with minimal oral inflammation (low number of oral PMNs). In addition, conditions were selected for preliminary studies (phenytoin-associated gingival overgrowth, smoking) in which the amount of myeloperoxidase may be increased in whole saliva supernatant.

Materials and Methods. *Myeloperoxidase assay.* The reaction conditions previously described for the estimation of chloroperoxidase (23) were used for the determination of myeloperoxidase. Incubation mixtures (22°C) made to 3 ml contained 300 μM potassium phosphate buffer (pH 2.75), 60 μM KCl, 6 μM H_2O_2, 0.3 μM monochlorodimedon (1,1-dimethyl-4-chloro-3,5-cyclohexanedione) and 0.1 ml of saliva or other sample. All reactants except the H_2O_2 were combined and allowed to set 2–3 min. The myeloperoxidase reaction was then activated by addition of the H_2O_2. The adsorbance loss at 278 nm resulting from the conversion of monochlorodimedon to dichlorodimedon (1,1-dimethyl-4,4-dichloro-3,5-cyclohexanedione) by the hypochlorite ions generated by myeloperoxidase was recorded with a Beckman Acta CIII spectrophotometer for 5 min during the initial linear change in optical density. One unit of myeloperoxidase activity was defined as the optical density decrease equivalent to conversion of 1 μM of monochlorodimedon to dichlorodimedon per minute with the above assay conditions.

Evaluation of myeloperoxidase assay. A series of preliminary experiments, including those summarized below, were performed to confirm the suitability of the chloroperoxidase assay for the quantitation of salivary myeloperoxidase. Known amounts of NaOCl were added to 3 ml of phosphate buffer (pH 2.75) containing only 0.3 μM of monochlorodimedon. An optical density decrease was observed at 278 nm as occurs during the conversion of monochlorodimedon to dichlorodimedon (23). The reaction was complete when the samples were mixed and returned to the spectrophotometer. Substrate conversion was stoichometric until 85–90% utilization. Addition of 100 μl of whole saliva supernatant (the usual volume in the reaction mixture) to the buffer–substrate mixture did not alter the reaction of NaOCl with monochlorodimedon. Inclusion of the saliva increased the pH of the reaction mixture by less than 0.1 pH units. The optical density change from the reaction of hypochlorite with substrate was approximately 10% less when 6 μM of H_2O_2 was included with the saliva. Lactoperoxidase in amounts similar to that in 0.1 ml of human saliva (milk, Sigma; 0.5–1.5 μg) gave no myeloperoxidase activity in the complete assay system. No decrease in 278-nm absorbance occurred when either the sample or H_2O_2 was excluded or when heat-inactivated saliva or PMN lysate was included in the reaction mixture.

Human saliva contains approximately 1 mM SCN^- (24, 25). This thiocyanate by serving as a substrate for lactoperoxidase and myeloperoxidase has the potential to give apparent or increased myeloperoxidase activity (reduction of monochlorodimedon absorbance). The assay conditions, however, were adjusted to eliminate interference from salivary lactoperoxidase and SCN^-. Lactoperoxidase, but not myeloperoxidase, is rapidly deactivated in the pH 2.75 phosphate buffer containing KCl. Thus, the brief incubation of the assay mixture prior to H_2O_2 addition eliminates any reaction with SCN^- catalyzed by lactoperoxidase. Maintenance of a high Cl^- to SCN^- ratio at low pH inhibits the oxidation of SCN^- by myeloperoxidase and H_2O_2. Thiocyanate in a concentration equivalent to 5 mM in saliva does not give greater apparent myeloperoxidase activity. Increasing the SCN^- concentration above 10 mM, however, produces additional loss of monochlorodimedon optical density.

PMNs isolated from freshly drawn blood (26) were used as a source of myeloperoxidase activity. Lysates of the PMNs were prepared by five cycles of freeze–thawing in physiologic

saline containing 0.1% hexadecyltrimethylammonium bromide. Supernatants were collected after centrifuging the lysates for 10 min at 10,000*g*. The optical density–time relationship given by amounts of a typical PMN lysate supernatant with activity similar to that of supernatants from low flow rate whole saliva is shown in Fig. 1a. The optical density decrease was linear throughout the 5-min assay time and was proportional to the amount of added PMN supernatant. Larger or lesser amounts of KCl and/or H_2O_2 in the incubation mixture did not give greater optical density changes from either PMN lysates or saliva supernatants. PMN myeloperoxidase activity also was measured in pH 2.6–4.0 McElvaine's citric acid-phosphate and pH 3.6–5.4 Walpole's acetate buffers. Neither of these buffers provided reaction conditions superior to that of pH 2.75 phosphate buffer. Amounts of a PMN lysate supernatant were added to 10 low flow rate whole saliva supernatants to give additional myeloperoxidase activity similar to that already present. Neither activation nor inhibition of PMN myeloperoxidase resulted from presence of the saliva. Intact PMNs were added to similar whole saliva supernatants. The mixtures were incubated for 15 min at room temperature and centrifuged for 10 min at 10,000*g*. Fifty to sixty percent of the PMN myeloperoxidase activity was recovered in the supernatants. The partial enzyme recovery resulted from incomplete disintegration following cell lysis of the PMN granules which contain myeloperoxidase. Mixtures were prepared containing 1.0, 0.50, and 0.10 mg of hydroxyapatite and the lysate from approximately 700,000 PMNs made to 2 ml with pooled low flow rate whole saliva supernatant (six samples). The mixtures were shaken for 15 min at 22°C and centrifuged at 10,000*g* for 5 min. The supernatants were analyzed for myeloperoxidase. Salivary sediment was collected from pooled low flow rate whole saliva (six samples). The salivary supernatant and sediment were recombined to give mixtures which contained the sediment from 1, 2, 5, and 10 ml of saliva in each milliliter of mixture. The same quantity of PMN lysate as above was added to each mixture. The mixtures were incubated and centrifuged and the supernatants analyzed for myeloperoxidase as described previously. Complete recovery of added myeloperoxidase activity was found in the supernatants following exposure to either hydroxyapatite or the salivary sediment.

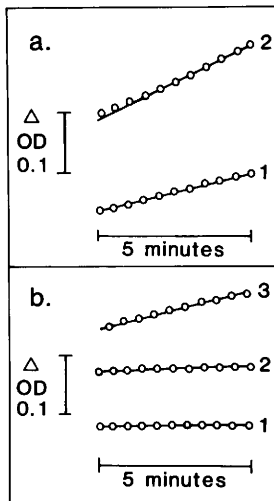

FIG. 1. Scale reproductions of optical density–time plots from incubation of monochlorodimedon with myeloperoxidase. See the text for complete incubation mixture. (a) Incubation with a PMN lysate supernatant from 125,000 cells, 0.0028 units myeloperoxidase (line 1), and 250,000 cells, 0.0057 units myeloperoxidase (line 2). (b) Incubation with low flow rate whole saliva supernatant from young adults with good oral health having relatively low (line 2, 0.4 units/100 ml) and high (line 3, 1.6 units/100 ml) myeloperoxidase activity. Line 1 (b) is a reagent blank (zero optical density-time change).

Collection, preparation, and assay of saliva samples. The analytical method for myeloperoxidase described above was applied to saliva samples collected in three experiments. In the first experiment four saliva samples were obtained in sequence from 21 (12 male, 9 female) young adults (nonsmokers). Assignment of individuals to this experimental group was based primarily on the presence of only minimal gingival inflammation (clinical indices not recorded). In addition, subjects were excluded who had oral ulcers or who were receiving drug therapy or had an oral or systemic disease known to affect oral microflora or salivary PMN function. All samples were collected between 2 and 3 PM. The subjects had not consumed food or fluids for at least 1 hr prior to sample collection. Whole saliva (3–5 ml) was first collected by the subject ex-

pectorating into a beaker. The subjects then rinsed their mouth for 1 min with each of 20 ml of physiologic saline and 20 ml of distilled water. The salt and water rinses were discarded. Whole saliva (5 ml) next was collected during chewing on paraffin. Whole saliva collected by expectoration and during chewing on paraffin are designated throughout this paper as low flow rate and moderate flow rate whole saliva, respectively. A Lashley cup was placed over one parotid papilla. Parotid saliva (1–2 ml) then was collected without additional stimulation. Finally, parotid saliva (3–5 ml) was obtained during sour lemon candy stimulation. The parotid saliva samples obtained without additional stimulation and with sour lemon candy stimulation are identified in this paper as moderate and high flow rate parotid saliva, respectively. Moderate flow rates of the first parotid saliva samples likely resulted from collection immediately following obtaining of paraffin-stimulated whole saliva. The whole (but not parotid) samples were clarified by centrifugation at 10,000*g* for 10 min.

In the second experiment, low flow rate whole saliva (3–5 ml) was collected at midmorning from 19 additional young adults (11 male, 8 female) all with minimal oral inflammation (controls) and from 6 young adult patients (3 male, 3 female) with Grades 1 and 2 phenytoin-associated gingival overgrowth [minimal to moderate severity (27)]. All the subjects with gingival overgrowth had moderate gingival inflammation [gingival index, 1 to 2 (28)]. For the final experiment, low flow rate whole saliva was obtained from 6 young adults (4 male, 2 female) with minimal gingival inflammation but who were chronic smokers. The samples from these two experiments were processed by the methods given above.

Results. The myeloperoxidase activity of the various saliva samples collected in the first experiment is given in Table I. Scale drawings of spectrophotometer tracings from low flow rate whole saliva supernatants with relatively high and low myeloperoxidase activity are shown in Fig. 1b. Samples which gave an optical decrease of less than 0.001/min (0.2 units/100 ml) were recorded as having zero activity. This lower limit of detection corresponds to the approximate optical density change given by 0.04 μg (0.4 μg/ml saliva) of lactoperoxidase in the thiocyanate oxidation method (1). Myeloperoxidase was detected in low flow rate whole saliva supernatants from 18 of the 21 subjects. In contrast, the enzyme was present, following a saline-water mouth rinse, in the moderate flow rate whole saliva supernatants from only 5 subjects. About half of the parotid saliva samples collected during moderate stimulation contained myeloperoxidase. However, only 4 of the 21 high flow rate parotid saliva samples included detectable myeloperoxidase. Myeloperoxidase activity, when present, was greater (with one exception) in low flow rate whole saliva supernatants and moderate flow rate parotid saliva than in the corresponding samples collected at greater flow rates. Fifteen of the 18 subjects with myeloperoxidase in low flow rate whole saliva supernatants had greater enzyme activity in low flow rate whole saliva supernatants than in moderate flow rate parotid saliva (the activities were equal in the other 3 individuals). The four high flow rate parotid saliva samples which contained myeloperoxidase, however, had more enzyme activity than the moderate flow rate whole saliva supernatants from the same individuals. Myeloperoxidase activity in low flow rate whole saliva supernatants and moderate flow rate parotid saliva from female subjects (1.40 ± 0.29 and 0.51 ± 0.10 units/100 ml) was significantly greater ($P < 0.01$ and 0.05) than that of the corresponding secretions from male donors (0.51 ± 0.10 and 0.17 ± 0.08 units/100 ml). Similar differences between males and females did not occur in the enzyme activity of moderate flow rate whole saliva supernatants and of high flow rate parotid saliva.

The myeloperoxidase activity of the low flow rate whole saliva supernatants from patients with gingival overgrowth appears bimodal (Fig. 2). No clear relationship between the severity of gingival overgrowth or degree of inflammation and the bimodal activity distribution was evident in the limited number of patients in this study. Consequently, for statistical comparison, the assumption was made that the apparent bimodal distribution was an artifact from a small number of samples. Application of the Wilcoxon two-sample rank-sum test (which does not assume a normal sample distribution) showed that the myeloperoxidase activity of low flow rate whole saliva supernatants from the 6 patients

TABLE I. MYELOPEROXIDASE IN VARIOUS TYPES OF SALIVA AND SALIVARY FLOW RATES FROM YOUNG ADULTS WITH MINIMAL GINGIVAL INFLAMMATION

		Myeloperoxidase (units/100 ml)			
		Whole saliva–flow rate		Parotid saliva—flow rate	
Subject No.	Sex	Low	Moderate	Moderate	High
1	F	1.0	0	0	0
2	F	1.6	0.2	0.8	0
3	F	2.0	0.6	0.4	0
4	F	0	0	0.6	0
5	M	0.8	0	0	0
6	M	0.8	0	0	0
7	F	1.0	0	0	0
8	M	0.4	0	0	0
9	M	0	0	0	0
10	M	0.4	0.4	0.4	0
11	M	0.6	0	0.4	0
12	M	0	0	0	0
13	F	0.4	0	0	0
14	F	2.8	0	1.2	0.6
15	M	0.8	0	0.8	0.4
16	M	1.0	0.4	0	0
17	M	0.6	0	0	0
18	M	1.0	0.2	0.8	0.4
19	F	1.8	0	0.8	0
20	F	2.0	0	0.8	0.6
21	M	0.4	0	0.4	0
Range		0–2.8	0–0.6	0–1.2	0–0.6
Number with activity		18	5	11	4
Mean		0.93	0.17[a]	0.35[b]	0.10[c]
SE Mean		0.16	0.04	0.09	0.04
		Flow rate (ml/min)			
Mean		0.48	1.63	0.29	0.94
SE Mean		0.05	0.17	0.04	0.10

[a–c] Significantly different from low flow rate whole saliva, [a]$P < 0.001$, [b]$P < 0.01$, and from moderate flow rate parotid saliva, [c]$P < 0.02$, Student's *t* test.

with phenytoin-associated gingival overgrowth (5.04 ± 1.84 units/100 ml) was significantly greater ($P < 0.01$) than that of the 19 controls (0.91 ± 0.26 units/100 ml) in Experiment 2 (Fig. 2). The myeloperoxidase activities from the two groups overlapped. However, none of the control samples had myeloperoxidase activity greater than the mean value from the gingival overgrowth group. Furthermore, only one of the samples from the gingival overgrowth patients was less than the mean of the controls. The mean myeloperoxidase activity (5.66 ± 1.79 units/100 ml) of low flow rate whole saliva supernatants from the individuals who were chronic smokers was significantly greater ($P < 0.01$, Wilcoxon two-sample rank-sum test) than that of either control group. A bimodal distribution was not evident in the individual activity values (2.2, 3.4, 4.0, 6.6, 6.8, and 11.2 units/100 ml) of this group.

Discussion. Most supernatants of low flow rate whole saliva from young adults with minimal oral inflammation contained peroxidase activity with the properties of myeloperoxidase. The major source of this peroxidase activity likely was myeloperoxidase from PMNs which migrated into the oral cavity. Other cell types which contain peroxidases that oxidize Cl^- to generate HOCl (monocyte myeloperoxidase, eosinophil peroxidase) are unlikely to be present in the oral cavity in numbers adequate to contribute significantly to oral

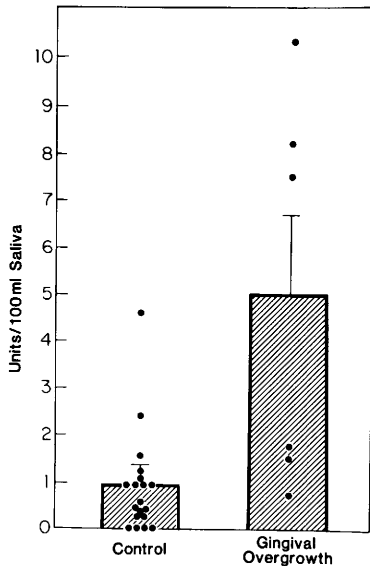

FIG. 2. Myeloperoxidase in low flow rate whole saliva supernatants from controls and from seizure patients with phenytoin-associated gingival overgrowth. The histograms give the group means and the standard error of the means.

peroxidase activity. The highest myeloperoxidase activity was in low flow rate whole saliva supernatants where PMN products have the greatest opportunity to accumulate. Furthermore, myeloperoxidase was not detected in the supernatants of most whole saliva samples collected at a moderate flow rate following a saline-water rinse which at least partially removed the PMNs and their disintegration products from the mouth. Mean flow rate differences between low and moderate flow rate whole saliva (Table I) only partially account for the different whole saliva supernatant myeloperoxidase activities (mean secretion rates, low and moderate flow rates, 0.45 and 0.28 units/100 ml, respectively).

Small quantities of myeloperoxidase entered the oral cavity of about half the subjects in parotid fluid. Parotid saliva from individuals without parotid disease is generally considered essentially free of bacteria or cells such as PMNs. The presence in parotid saliva of the PMN product myeloperoxidase, nevertheless, shows that discharge of PMN components into the gland fluid is common in subjects without known parotid disease. Some enzyme may have washed from the mucosal surface or the duct orifice into the samples. The exposed tissue surface, however, is small. Therefore, it is unlikely that sufficient enzyme washed into the samples to account for the parotid saliva myeloperoxidase activity. In progressing from a moderate to high level of stimulation, the mean amount of parotid saliva myeloperoxidase decreased in proportion to the increased flow rate (Table I). The concentration of myeloperoxidase may be even greater in parotid saliva collected at low than at moderate flow rates. Precise evaluation of the changes in parotid saliva myeloperoxidase activity with flow rate is complicated by the low or undetectable levels of enzyme activity in most samples. Additional amounts of myeloperoxidase probably are present in parotid saliva during certain clinical conditions. For example, during the initial flare up period of chronic recurrent parotitis, lysozyme and lactoferrin, products of both the parotid gland and PMNs, were found in high concentration in parotid saliva (29). Myeloperoxidase also was present, but in undetermined amounts (29).

The myeloperoxidase in low flow rate whole saliva supernatants appears to be a significant portion of the enzyme in the PMNs which enter the oral cavity. Peripheral blood PMNs isolated from four subjects contained an average of 0.019 units myeloperoxidase/10^6 cells (0.014, 0.023, 0.017, and 0.022 units/10^6 cells). Low flow rate whole saliva supernatant (flow rate, 0.5 ml/min; 0.93 units myeloperoxidase/100 ml) thus included approximately 25% of the enzyme present in the 10^6 PMNs/min which enter the mouth of individuals with good oral health (13, 16, 30; this laboratory, unpublished). Myeloperoxidase also apparently makes an important contribution to the total low flow rate whole saliva supernatant peroxidase activity. Myeloperoxidase in a PMN lysate supernatant was determined by the procedure (1) previously used in this laboratory to express total salivary peroxidase activity based on lactoperoxidase standards. This analysis showed that the 0.93 units myeloperoxidase/100 ml of low flow rate whole saliva supernatants from subjects with good oral health represents 15–20% of the total whole salivary peroxidase previously determined as lactoperoxidase [7.9 μg/ml, (9)].

Segregation of the myeloperoxidase activities from the first experiment into those from

male and female subjects indicates a possible sex related difference in the quantity of the enzyme in saliva. Peroxidase in whole saliva may be subject to hormonal variation as indicated by reports of changes in peroxidase activity associated with ovulation (7, 8). Saliva collection in the present experiments was random and, thus, the observed male–female differences are not likely related to ovulation. The greater myeloperoxidase activity in the female subjects does indicate that a larger PMN contribution to saliva occurred through greater PMN migration into the oral cavity and/or more rapid cell lysis. Additional experiments which include measurement of the number of PMNs entering the oral cavity, the clinical status of the gingiva and the myeloperoxidase activity of gingival fluid and saliva are required to confirm and explain the observed differences for myeloperoxidase activities between male and female subjects.

The analyses of the samples from patients with phenytoin-associated gingival overgrowth and from individuals who smoke demonstrate that low flow rate whole saliva supernatant myeloperoxidase likely is increased in certain circumstances. More peroxidase activity (measured as lactoperoxidase) in low flow rate whole saliva supernatants of patients with phenytoin associated gingival overgrowth previously was reported by this laboratory (9). The conclusion made in the earlier study that PMNs furnished at least a portion of the additional peroxidase is supported by the present data. This conclusion, however, must be considered as only tentative because of the small numbers of samples and the apparent bimodal distribution of myeloperoxidase activity in the gingival overgrowth group. Calculations made in a manner similar to those performed with the control subjects disclosed that 40–50% of the peroxidase activity in low flow rate whole saliva supernatants previously measured as lactoperoxidase in patients with Grade 3 (severe) gingival overgrowth [20.3 ± 4.8 μg/ml, (9)] was from myeloperoxidase. The mean SCN^- concentrations (159 ± 75.5 mg/liter) in supernatants of paraffin-stimulated whole saliva from 27 smokers was two to three times that of controls (31). This quantity of SCN^-, as discussed previously, is inadequate, to interfere with determination of myeloperoxidase activity. Greater amounts of salivary myeloperoxidase also may occur in low flow rate whole saliva supernatants in other conditions. Myeloperoxidase may accumulate during sleep, when salivary flow is very low with consequent slow removal of PMN products. It is likely that a greater amount of myeloperoxidase is present in low flow rate whole saliva supernatants of subjects with severe gingival inflammation because of the increased number of PMNs which enter the oral cavity (10–16).

The biologic significance of the myeloperoxidase in saliva is unknown. Myeloperoxidase, as well as lactoperoxidase, interacts with SCN^- to give $OSCN^-$ (32). H_2O_2, however, is the limiting factor for generation of $OSCN^-$ in human saliva (33). Thus, the presence of myeloperoxidase may not result in production of additional amounts of the antimicrobial metabolite, $OSCN^-$. Myeloperoxidase might have an indirect effect on the generation of $OSCN^-$ by consumption of H_2O_2 during the formation of OCl^-. This reaction, however, may not be of significance because of the much greater affinity of myeloperoxidase for SCN^- than for Cl^- at salivary pH. Nevertheless, it is evident that the presence of myeloperoxidase in saliva must be considered during investigation of the salivary peroxidase system. Such considerations are particularly important in circumstances such as gingival overgrowth or smoking where the myeloperoxidase contribution to total salivary peroxidase activity appears greatly increased.

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