Turku sugar studies XII

The effect of the diet on oral peroxidases, redox potential and the concentration of ionized fluorine, iodine and thiocyanate

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Detailed biochemical analyses of peroxidases in saliva, plaque and gingival exudate samples were carried out in view of the preliminary findings that the peroxidase activity of centrifuged oral fluid was considerably higher in the xylitol group than in the fructose or sucrose groups. Chromatographic experiments revealed the activity which was increased due to the intake of xylitol, to be attributed to the involvement of the salivary lactoperoxidase, and not to enzymes formed in plaque or leucocytes. There were no significant differences between the sugar groups in the concentration of thiocyanate ions (mean 92 mg/l) and ionized iodine (mean 1.6 μg/l), but the concentration of ionized fluorine in saliva was lower in the xylitol group (0.128 mg/l) than in the other groups (0.150 mg/l). There were no clear differences in the salivary redox potential between the sugar groups. It is evident that various sugars selectively affect the enzyme and other production of the salivary glands. Xylitol-induced elevation of the salivary lactoperoxidase activity and the cariostatic properties of xylitol may partly be interrelated phenomena due to the antibacterial properties of lactoperoxidase.

Key-words: Sucrose; fructose; xylitol; peroxidases; fluorine; iodine; thiocyanate; oxidation-reduction

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Peroxidase activity in human whole saliva is largely derived from the salivary glands, leucocytes and bacteria. The actual peroxidase of the saliva proper, the lactoperoxidase, has been suggested to act as an antimicrobial factor in the oral cavity, provided that certain auxiliary compounds are available (*Hoogendoorn & Moorer*, 1973; *Koch, Edlund & Hoogendoorn*, 1973; *Hugoson et al.*, 1974; *Morrison & Steele*, 1968). Such compounds include hydrogen peroxide, a compound to be oxidized, and SCN^- or I^- ions. This entity can be called the lactoperoxidase system.

The biochemical studies of the present trial showed that after a few month's dietary period the overall peroxidase activity of centrifuged oral fluid exerted an interesting difference between the three sugar groups; in the saliva of the xylitol-consuming test persons the enzyme activity seemed to be considerably higher than in the other groups. These preliminary findings led to the present series of chromatographic experiments and to the investigation of the peroxidase activity during the rest of the trial. A part of the biochemical data and a description of pertinent literature have also been published elsewhere (*Mäkinen, Tenovuo &*

Scheinin, 1975). The present paper also provides information about the assays of the auxiliary compounds mentioned above (SCN^- and I^- ions), as well as about the salivary redox potential. For the sake of comparison, the concentration of another free halide ion, F^-, was also determined.

MATERIAL AND METHODS

1. *General methods*

The principal details of the materials and methods of the present trial have been earlier described (*Mäkinen & Scheinin*, 1975a, b). The first peroxidase assays of the samples were carried out approximately 3.5 months after the initiation of the trial. After this the subjects were analyzed at the other occasions indicated in the Results section of this paper. In general, all the samples analyzed for peroxidase and catalase activity in this study were stored at —20° C after collection before use (up to 3—4 weeks). It was found, however, that the peroxidase activity of saliva, plaque and exudate was not to any noticeable extent affected by this storage. Furthermore, the three sugar groups were in all cases comparable, because analyses on respective materials were performed approximately at the same time, regardless of the length of storage. For all other tests of this study fresh samples were used.

2. *Determination of peroxidase and catalase activity*

The peroxidase activity was determined with the guaiacol method as suggested by *Chance & Maehly* (1964). The specific activity was calculated as the ratio of 60 sec to the reaction time in seconds to reach an extinction of 0.050 in a reaction mixture described in the above source. The value obtained was expressed per mg protein [*i.e.* in (60 sec/*a* sec)/mg protein where *a* is the reaction time.] Catalase was assayed spectrophotometrically as suggested by *Lück* (1963) with the supernatant fluid of whole mouth saliva. All enzyme assays were performed with a Hitachi Perkin Elmer UV-VIS Spectrophotometer.

3. *Determination of proteins*

Proteins were determined according to the *Lowry*'s method (*Lowry et al.*, 1951) using bovine serum albumin as standard (Sigma Chemical Company, Los Angeles, Cal., USA).

4. *Chemicals*

All chemicals were of analytical grade. Guaiacol was a product of BDH Chemicals Ltd. (Poole, England). Other chemicals were obtained, unless otherwise mentioned, from E. Merck AG (Darmstadt, Germany). The water used in the solutions of this paper was distilled and passed through an ion exchange column. The specific resistance of the water was approximately 1 MΩ-cm.

5. *Collection and treatment of samples*

a. General conditions. The collection of saliva, plaque and exudate samples has been described earlier (*Mäkinen & Scheinin*, 1975b; *Paunio, Mäkinen & Scheinin*, 1973). It may be mentioned, however, that the plaque and other samples were usually obtained simultaneously with the clinical inspections. The samples were collected between 8 am and 3 pm. The test groups were comparable with each other with regard to the time of day when the collections were performed. The subjects were requested to refrain from all oral hygiene procedures for 12—24 hours before the sample collections. The sample collections

were started with a 5 min plaque collection *in situ* after which the test persons provided a 10 ml sample of oral fluid, followed by the collection of the exudate.

The number of subjects involved in each sugar group was in the beginning of the trial as follows: sucrose (S), 35; fructose (F), 38; xylitol (X), 52. All details describing the subjects and the general treatment of the sugar groups and the dietary regimen has been provided earlier (*Mäkinen & Scheinin*, 1975a).

b. Plaque. The plaque samples were collected *in situ* from all available surfaces as described earlier (*Scheinin & Mäkinen*, 1971). Each plaque sample was immediately weighed with a Mettler Type HI5 balance.

The plaque samples were stored after the collection and weighing in cold (+4° C) and pooled at the end of each collection day to three pools. The pools were then frozen at —20° C. Approximately within three weeks the three complete plaque pools were gradually formed. The material was then thawed and mixed with 5.0 ml of cold 0.154 M NaCl solution (0.9 %). The mixtures were thoroughly stirred for 3 min with a glass rod and centrifuged for 10 min at 23500 × *g* with a Sorvall RC-2B refrigerated centrifuge. The clear supernatant fluids were kept at —20° C.

The pellets remaining after the above centrifugation were also pooled and stored at —20° C untill they were thawed and treated with an ultrasonic disintegrator as described later. The plaque material was accordingly divided into an aqueous extract and a water insoluble (and sedimentable) phase of which the latter was sonicated.

c. Saliva. The subjects gave the 10 ml oral fluid samples using paraffin stimulation. The samples were kept for 2—3 hours in an iced water bath (+4° C) before all samples collected on the same day were centrifuged as above. The supernatant fluids were kept overnight at +4° C for the chemical analyses on the subsequent morning. The pellets obtained were pooled and stored at —20° C for later sonication and analyses. In the pooling of the pellets 75 ml of a 0.154 M NaCl solution was used (at +4 ° C) for the material obtained in the sucrose and fructose groups (100 ml for the xylitol group).

d. Sonicated salivary sediment. All ultrasonic treatments were carried out with a MSE Ultrasonic Disintegrator (Modell 100 W, Measuring & Scientific Equipment, Poole, England) using a probe with 15 mm end diameter and 25 ml jacketed vessels equipped with side-ports for coolant circulation. The samples were treated for 10 min in 20 ml portions. The original volume of suspension solution used in each sugar group was mentioned in the preceding paragraph. The resulting mixtures were centrifuged for 15 min at 45000 × *g* and the supernatants were frozen at —20° for later analyses.

e. Sonicated plaque sediment. For the pooled plaque material collected *in situ* the procedure was in principle the same, but a probe with an end diameter of 9 mm was used. The volume of the suspensions treated in this case was 5 ml. In the sonication, the plaque material representing each sugar group (the material described in paragraph *b* above) was suspended in 10 ml of cold 0.154 M NaCl solution (the whole sample was thus sonicated in two separate portions).

6. *Chromatography*

Ion exchange and molecular exclusion chromatography was performed according to *Peterson & Sober* (1962), and to the

instructions of the supplier of the gels, respectively. The gels used were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and from Calbiochem (Los Angeles, Cal., USA). For ion exchange chromatography on DEAE- and CM-cellulose a special fraction (230—270 mesh) was sieved from the commercial preparation (Schleicher & Schüll, Dassel/Kr., Einbeck, Germany). Other details are given in the Results section of this paper. The samples for CM-cellulose chromatography from whole saliva supernatant fluid and the corresponding sediment were made as follows. After thawing, the pooled samples representing the three sugar groups were centrifuged for 10 min at 23500 × *g* in cold (+4°C). 50 ml of the pooled supernatant fluids from each test group was concentrated by freeze-drying. The solid residue was dissolved in 5.0 ml of cold 0.01 M ββ-dimethylglutarate buffer, pH 7.2. Evident turbidity was spun down. 1.0 ml of an aqueous Blue Dextran solution (0.1 %) was added and the 6.0 ml sample was passed through a Sephadex G-25 (Coarse) column (5.5 × 32 cm) with the aid of the above buffer. 27—29 ml was collected from the column. From the resulting desalted solution 27 ml was applied in each case on the CM-cellulose column. The preparations of the samples of the sonicated whole saliva sediments were treated before CM-cellulose chromatography as mentioned above. Consequently, approximately 50 ml of the crude peroxidase preparation resulting from the sonication step was treated with the Sephadex G-25 column.

In addition to the above mentioned steps on CM-cellulose, the pooled samples of gingival exudate and the supernatant fluids of sonicated plaque (collected *in situ*) were also analyzed on DEAE-cellulose columns. With the plaque preparations the pretreatment of samples was in principle as for CM-cellulose chromatography mentioned above. Pooled and centrifuged exudate samples were applied on the DEAE-cellulose columns without pretreatment. The detailed procedure is presented below.

Gingival exudate. The analysis of peroxidase activity in the gingival exudate samples was performed after one year of the dietary regimen. The number of subjects involved in the sugar groups was: S and F, 33, X, 51. Because ten filter paper strips were used for each test person, the final number of strips in each test group was: S and F, 330 strips, X, 510 strips. It should be mentioned that the number of subjects was not exactly the same in all experiments carried out in the course of the trial, e.g. due to occasional loss of samples, discontinuation or illness of the subjects. The paper strip mass of the X-group was suspended in 30 ml of cold (+4°C) 0.01 M ββ-dimethylglutarate buffer, pH 7.2 (25 ml in the F- and S-groups). The mixtures were stirred with a glass rod in cold for 35 min and centrifuged for 20 min at 43500 × *g* (18000 rpm). The supernatant fluids were removed and the remaining filter paper sediment was suspended in the above buffer (4.0 ml in the case of S- and F-groups and 5.0 ml in the case of the X-group). After 15 min stirring with a glass rod in cold the mixtures were centrifuged as above. The resulting supernatant fluids were pooled with the above larger supernatant fluids. The three exudate pools were concentrated with an Amicon Ultrafiltration Apparatus (membrane UM2OE) in the following way:

S-group: 29 ml → 12 ml
F-group: 25 ml → 14 ml
X-group: 25 ml → 15 ml

The resulting clear preparations were

directly applied on a DEAE-cellulose column.

Sonicated plaque. The procedure with the supernatant fluids of sonicated plaque was as follows: 2.5 ml aliquots of the supernatant fluids were mixed with 2.0 ml of an aqueous Blue Dextran solution (0.1 %). The solution was passed through a Sephadex G-25 (Coarse) column (5.5 × 32 cm). The desalted solutions representing the three sugar groups were concentrated to 10 ml with the Amicon Ultrafiltration Apparatus as above. The resulting preparations were applied on a DEAE-cellulose column.

7. *Determination of ionized fluorine and iodine*

Ionized fluorine and iodine of individual samples of centrifuged whole mouth saliva were determined at the end of the two year study electrometrically with the aid of a calomel and fluoride ion and iodide ion electrodes, respectively (Beckman, No. 445 and 39606). Sodium fluoride and potassium iodide were used as standards. In the assay of ionized fluorine and iodine the Radiometer pH Meters 26 and 28 were used, respectively. The assay of both compounds was performed simultaneously in the same 25 ml vessel with two calomel electrodes and the respective ion specific electrodes, using approximately 8 ml saliva samples. The assays were performed at +22°C 2—6 hours after the collection of the samples. The three sugar groups were comparable with regard to the time when the assay of individual samples was performed after collection.

8. *Determination of thiocyanate*

Thiocyanate ions of individual samples of centrifuged whole mouth saliva were determined at the end of the two year study colorimetrically according to *Powell* (1945). The assay of thiocyanate ions was performed on the following day after the collection of samples (stored at +4°C overnight).

9. *Determination of redox potential, pH and rH*

After approximately 16.5 months dietary period the whole mouth saliva samples of the test persons were subjected to detailed analyses with regard to redox potential (E_h). The measurements were carried out immediately after the collection of a 10 ml saliva sample using paraffin stimulation. The flow rate of saliva was simultaneously determined. Redox potential was measured with a Radiometer pH Meter (Type 28), using a platinum and a calomel electrode according to the manuals of the supplier. The pH of the samples was subsequently measured with a glass and calomel electrode. E_h and pH were determined at +22°C.

The measured potential difference, E, was used as an indication of the redox potential, E_h. rH (or pH_2, hydrogen pressure above the solution) was calculated from $rH = 2pE + 2pH$, where pE is the redox exponent, defined as $pE = E_h/0.1984\ T$, T being the absolute temperature, and E_h being measured in mV. E_h was computed from $E_h = E + E_{\text{reference electrode}}$. For $E_{\text{reference electrode}}$ a value of 240 mV was used. Due to several practical reasons the redox potential was measured in open semicone-shaped plastic vessels (25 ml) immediately after the collection of the saliva samples. The surface area and volume of the samples in the vessels were approximately the same throughout the study. The conditions were made as similar as possible for all measurements. The potentials were

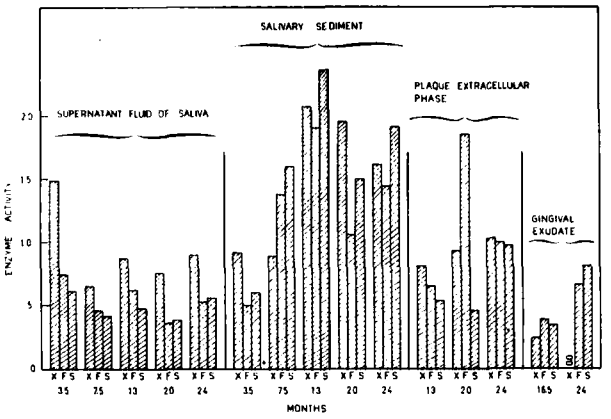

Fig. 1. Specific peroxidase activity of various samples of oral origin. Note that it was only in the supernatant fluid of whole saliva which constantly displayed the highest activities in the xylitol group.

recorded at 5 sec and at 1, 2 and 3 min after the formation of the contact between the solution and the electrodes.

RESULTS

1. *Peroxidase activity*

The peroxidase assays were carried out approximately 3.5, 7.5, 13, 20 and 24 months after the start of the regimen. Saliva samples were analyzed at all analysis periods indicated. Other biological samples of oral origin were included in later analyses when it became evident that their possible contribution to the overall peroxidase activity should be taken into account.

Fig. 1 shows the peroxidase activity in the biological materials indicated. Without exception, the supernatant fluids of whole mouth saliva displayed the highest specific peroxidase activity in the X-group. With the two other sugar groups the activity was more similar and clearly lower than in the X-group samples. Fig. 1 shows that the sonicated sediment material, corresponding to the above supernatant fluids, exerted considerable variation in peroxidase activity between the different analysis periods and between the three sugar groups. There was, however, no clear trend in favour of any of the sugar groups with regard to this phenomenon. The variation between the analysis periods was considered to be a result of the following possibilities:

1) Difficulties in having the samples to be sonicated in an exactly identical manner, (however, see *Mäkinen & Scheinin*, 1975b).
2) Seasonal and other differences in the microbial and other composition of the sediment material between the test periods.

The aqueous extracts of the bacterial plaque and gingival crevice fluid were analyzed three (13, 20 and 24 months) and two (16 and 24 months) times, respectively. For the plaque extracellular fluid the activities shown in Fig. 1 indicate that there were also slightly higher values in the X-group than in the other sugar groups. It is to be emphasized that the aqueous plaque extract always contains salivary ingredients. Consequently, these slightly higher values in the X-group may have resulted from the higher peroxidase activity of saliva in this same sugar group.

Fig. 1 indicates that the saliva of the subjects on X-diet for a longer period displayed a higher overall specific peroxidase

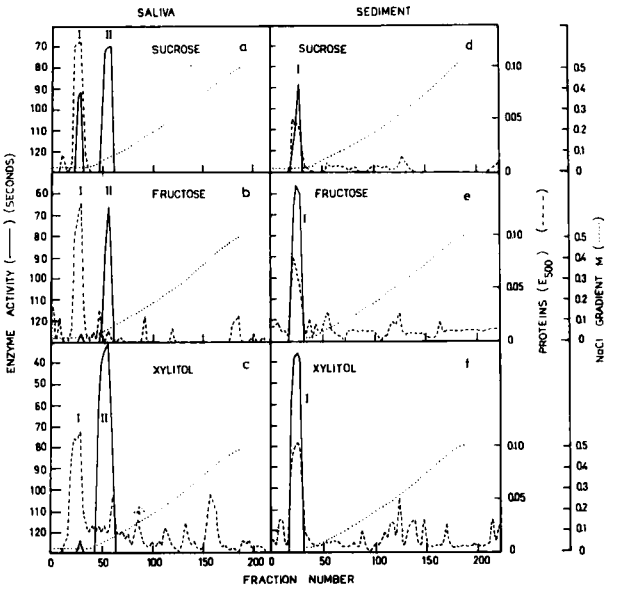

Fig. 2. CM-cellulose chromatography of the supernatant fluid of human whole mouth saliva peroxidases (a, b, c) and that of sonicated whole saliva sediment peroxidases (d, e, f), determined according to the guaiacol method. The pooled samples were obtained from persons on either sucrose, fructose, or xylitol diet. Sample: 27 ml of a desalted crude peroxidase solution (preparation mentioned in the text); Column: 1.8 × 38 cm; Elution buffer: 0.01 M ββ-dimethylglutarate buffer, pH 7.2; NaCl gradient: linear from 0 to 1.0 M; Mixing volume: 250+ 250 ml; Hydrostatic pressure in packing and elution: 120 cm; Temperature: +4° C. Enzyme activity is given in seconds as mentioned in the Methods section. Protein is given in extinctions (Folin Ciocalteu method). The form of the NaCl gradient was determined by atomic absorption spectrophotometry.

activity than the other samples studied. For gingival crevice fluid the specific overall peroxidase activity was lowest in the X-group. At this particular stage of study the separate and individual contribution of the true salivary lactoperoxidase, the leucocyte myeloperoxidase and the possible pseudoperoxidase activity could not be evaluated.

The above mentioned results led to various fractionation and characterization experiments in which the reason for the elevation of the peroxidase activity in the oral fluid was elucidated. It appeared that the component peroxidase occurring in the human oral fluid could be separated by ion exchange chromatography using a linear sodium chloride gradient. Fig. 2a shows that with the supernatant fluid of whole saliva two peroxidases (I and II) were obtained. Peak I was not absorbed to CM-cellulose in the conditions involved. As indicated by the other representative chromatograms (Figs. 2b, 2c), Peak I was lacking practically totally in the two other groups (F & X). It was also apparent that the peroxidase of the second enzyme peak was detached from the column at approximately 0.07 M NaCl. The peroxidase activity of this peak (Peak II) was clearly higher in the X-group than in the sucrose and fructose groups. Repeated experiments at different analysis and collection periods showed that Peak II in the X-group was constantly higher than the sum of the two peroxidase peaks in Figs. 2a (S) or 2b (F). The same result was obtained when the enzyme activity was expressed as specific activity. Consequently, it was very likely that this particular peroxidase peak caused the elevated overall peroxidase activity in the crude preparations (shown in Fig. 1).

When the corresponding sonicated whole saliva sediment preparations were fractionated through CM-cellulose columns in conditions identical to those indicated above, only one single higher peroxidase peak (Peak I) appeared in the fractions (Figs. 2d, e, f). Consequently, it

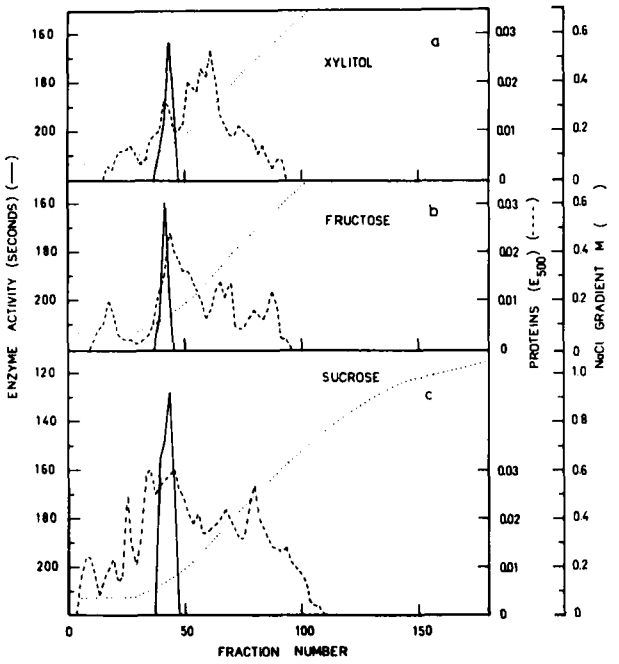

Fig. 3. DEAE-cellulose chromatography of peroxidases of pooled samples of gingival crevice fluid. Column: 1.7 × 18 cm; Elution buffer: 0.01 M ββ-dimethylglutarate buffer, pH 7.2. Mixing volume of the 0—0.1 M NaCl gradient: 150 ml + 150 ml. Samples: 14 ml of concentrated gingival crevice fluid preparation (see text). Fraction volume 1.5 ml. Temperature: +4 C.

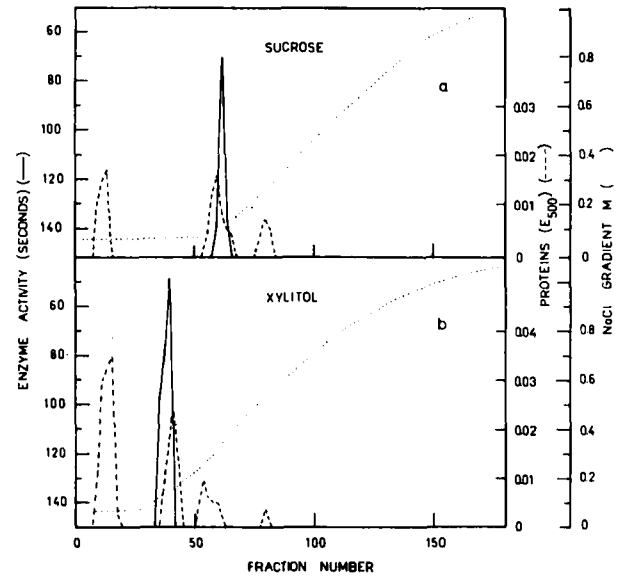

Fig. 4. DEAE-cellulose chromatography of the supernatant fluid of sonicated and pooled plaque material. Sample: 37 ml. Other details as for Fig. 3.

was considered likely that the lower peak (Peak I) in Fig. 2 (a, b, c) was also derived from the cellular or sedimentable fraction of whole mouth saliva. At this stage it was possible to assume that Peak II represented the true salivary lactoperoxidase, whereas Peak I most likely corresponded to bacterial enzymes, the leucocyte myeloperoxidase and/or some pseudoperoxidase activity. These conclusions were considered

Table I. *Specific peroxidase activity of Peaks I and II obtained in CM-cellulose chromatography of pooled samples of the supernatant fluid of whole saliva and of the supernatant fluid of sonicated salivary sediment. Representative values obtained from samples collected 7.5, 13 and 24 months after the start of the test. S, sucrose; F, fructose; X, xylitol*

Months	7.5	13	24	Sample
Sugar group				
S	5.0	15.8	13.6	Peak II (lactoperoxidase) of the supernatant
F	10.9	28.1	—*	fluid of whole saliva
X	26.5	118.0	30.0	
S	2.9	14.9	5.1	Peak I (myeloperoxidase, bacterial peroxidase
F	2.5	9.5	4.4	and/or pseudoperoxidase) of the supernatant
X	3.1	6.7	2.5	fluid of whole saliva
S	2.2			Peak I (myeloperoxidase, bacterial peroxidase
F	6.1			and/or pseudoperoxidase of sonicated salivary
X	1.4			sediment)

* Activity too low for reliable measurements.

rather well-grounded, because the possible sources of peroxidase activity in the human oral cavity are generally known. The fractionation pattern indicated in Fig. 2 was constantly obtained throughout the trial with each of the material tested.

When gingival exudate and samples of sonicated bacterial plaque (the sediment corresponding to plaque aqueous extract) were chromatographed under identical conditions on DEAE-cellulose, one peroxidase peak appeared in the chromatograms (Figs. 3 & 4). The respective enzyme was absorbed to DEAE but not to CM-cellulose. Thus this peroxidase was not identical to Peak II of the chromatograms representing the supernatant fluid of whole mouth saliva.

The active fractions shown in the chromatograms of Fig. 2 were pooled and the resulting preparations were analyzed for specific peroxidase activity (Table I). It appeared that Peak II (tentatively designated at this stage as the salivary lactoperoxidase) was clearly the most active of all the samples tested. For Peak I (representing most likely myeloperoxidase, pseudoperoxidase and/or bacterial peroxidase activity) the situation was either the opposite or the activity in the X-group was only slightly higher. In whole mouth saliva sediment the peroxidase activity in Peak I was lowest in the X-group. The results of Table I supported the idea of the activity of Peak II being the reason for the higher overall peroxidase activity attained in the X-group saliva when compared to the other two test groups.

The molecular weight of the enzymes contained in Peak I and Peak II was determined using molecular exclusion chromatography on Sephadex G-100 Superfine and Bio-Gel P-300. The results are shown in Figs. 5—8. For the peroxidase represented by Peak I a value of 152000 was obtained. For Peak II (most likely representing the salivary lactoperoxidase) a value of 73500 was achieved. The gel permeation chromatography also revealed in all test groups (using saliva supernatant fluid) a peroxidase (Peak III) with a molecular weight of 12000. The

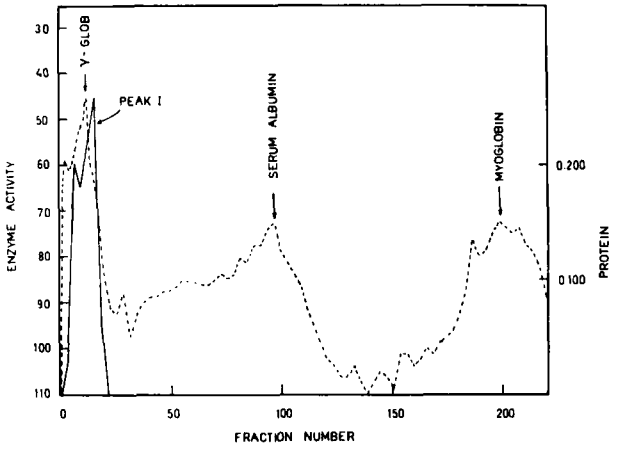

Fig. 5. Determination by molecular permeation chromatography on Bio-Gel P-300 of the molecular weight of a peroxidase (Peak I) revealed in CM-cellulose chromatography. Column: 1.6 × 140 cm; Elution buffer: 0.01 M ββ-dimethylglutarate buffer, pH 7.2; Sample: 2.0 ml of Peak I, supplied with 0.5 ml of Blue Dextran solution and 2.0 mg of the reference proteins indicated; Hydrostatic pressure: 15 cm; Temperature: +4° C. The subsequent plot is shown in Fig. 6.

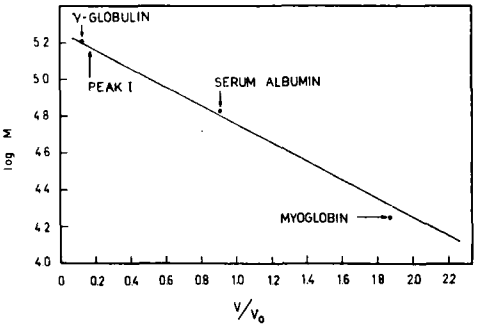

Fig. 6. Determination of the molecular weight of the peroxidase of Peak I. MW: 152000. Other details as in Fig. 5. $V = V_e - V_o$; $V_o = 106$ ml.

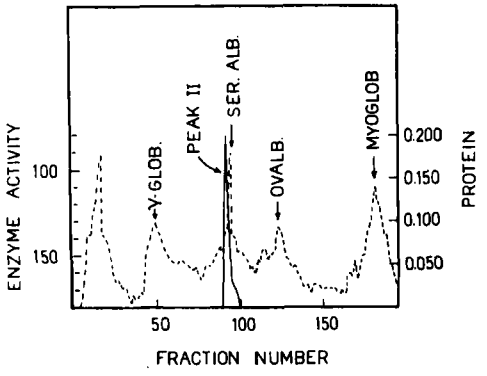

Fig. 7. Determination by molecular permeation chromatography on Sephadex G-100 Superfine of the molecular weight of a peroxidase (Peak II) revealed in CM-cellulose chromatography. Sample: 2.0 ml of Peak II. Other details as in for Fig. 5.

nature of this activity was not elucidated and its contribution to the differences shown between the sugar groups was considered almost nil. The eventual role of catalase and some other factors to the elevated peroxidase activity were examined.

Experiments carried out with the 24 mouth saliva and other samples gave the following results:

a. The catalase activity. The centrifuged supernatant fluids of whole mouth saliva of the test persons, obtained at the end of the trial, were tested for catalase. The material studied represented pools of the three sugar groups. The catalase activity of centrifuged whole mouth saliva was practically nil. It was not considered necessary to test other samples.

b. The effect of cyanide ions. The effect of cyanide ions (added as KCN) on the activity of the CM-cellulose peaks of whole saliva is shown in Table II. It appeared that 1.0 mM CN^- ions inhibited totally the activity of Peak II (lactoperoxidase), but the activity of Peak I was inhibited to a lower extent (by 95 per cent). This result was obtained with material derived from the sucrose and fructose group. In the xylitol group the situation

Table II. *The effect of cyanide ions (1.0 mM, added as KCN) on the peroxidase activity of Peaks I and II (given in per cent of inhibition)*

Sugar group	Rate Peak I	Inhibition %	Rate Peak II	Inhibition %
X	0	100	0	100
F	0.23	94.8	0	100
S	0.22	95.7	0	100

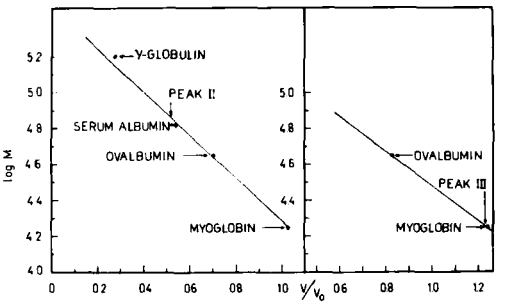

Fig. 8. Determination of the molecular weight of the peroxidase of Peak II. MW: 73500. Other details as in Fig. 7. $V = V_e - V_o$; $V_o = 75$ ml.

for Peak II was as above, but the activity of Peak I was inhibited by 100 per cent.

c. The activity of peroxidase in serum. Pooled serum samples representing the test groups did not display measurable peroxidase activity.

d. Effect of 4 per cent sugar on the peroxidase assay. Xylitol, fructose and sucrose (tested at 4 per cent concentration in the reaction mixtures) did not affect the peroxidase activity. The concentration tested was deliberately rather high, because such amounts of sugars often occur in the oral cavity.

2. *Ionized iodine and fluorine*

The ionized iodine and fluorine of the centrifuged whole mouth saliva samples were determined electrometrically at the end of the study. Results are shown in Table III for the three sugar groups. There was no clear difference between the groups with regard to the measurements of iodide. However, the concentration of fluoride ions was lower in the xylitol group when compared to the values obtained in the two other test groups.

3. *Thiocyanate*

Thiocyanate was determined colorimetrically with the centrifuged whole mouth saliva samples of all test persons at the end of the trial. In line with ionized iodine, the concentration of SCN^- ions was almost equal in all three sugar groups. (Table III).

There was an interesting indirect correlation with regard to the concentrations of ionized iodine and thiocyanate ions

Table III. *Concentration of thiocyanate and ionized iodine and fluorine in centrifuged human whole mouth saliva. The values are means of determinations on individual test persons performed at the 24 month phase*

Test group	Thiocyanate				Iodide				Fluoride			
	mg/l		$M \times 10^3$		mg/l		$M \times 10^6$		mg/l		$M \times 10^6$	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
Sucrose	89	50	1.5	0.9	1.62	1.13	12.8	8.9	0.15	0.10	8.0	5.3
Fructose	105	75	1.8	1.3	1.54	1.99	12.1	7.8	0.15	0.07	7.9	3.8
Xylitol	83	53	1.4	0.9	1.65	1.08	13.0	8.5	0.13	0.50	6.7	2.5

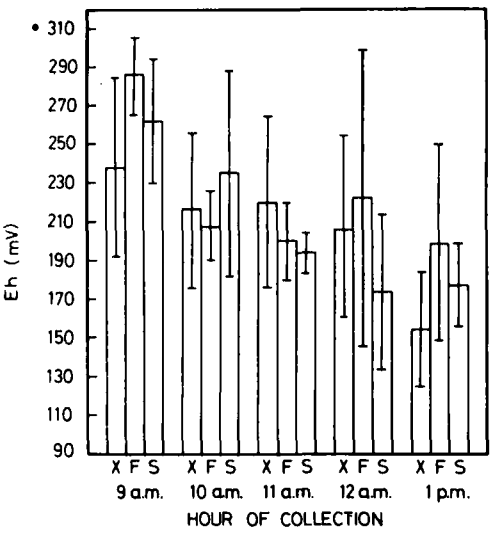

Fig. 9. Correlation between E_h and the hour of collection.

(Table III). The higher the amount of thiocyanate in centrifuged oral fluid, the lower the concentration of ionized iodine was.

4. *Redox potential*

The redox potential of uncentrifuged whole mouth saliva of all test persons was determined approximately 16.5 months after the start of the dietary period. Results are shown in Table IV and Fig. 9. The measurements of the redox potential indicated that the time of the day when the samples were collected, influenced the results obtained. Therefore, each sugar group was divided into three subgroups according to the time of the day the collection and measurements were performed (Table IV). The results showed that the redox potential (E_h), as well as rH and ΔE, were highest in the morning and decreased during the day. The difference between the subgroups I and III in each sugar group was remarkably great. On the other hand, there were no clear differences between the three sugar groups. It was also observed that the volume of the sample did not have any noticeable effect on the values. The average times needed for the collection procedure in each group are shown in Table IV. The differences between these values were so small that they had no remarkable effect on the results obtained.

In the conditions used (open vessels) the redox potential altered towards more

Table IV. *The redox potential of human whole saliva (determined immediately after collection of a 10 ml sample by paraffin-chewing). The three sugar groups were each divided into three subgroups according to the time of day when the samples were collected. Subgroup I, from 8 a.m. to 10 a.m.; Subgroup II, from 10 a.m. to 12 a.m., Subgroup III, from 12 a.m. to 2 p.m.*

Sugar Group	XYLITOL						FRUCTOSE						SUCROSE					
Sub-group	I(n=14)		II(n=29)		III(n=7)		I(n=13)		II(n=14)		III(n=10)		I(n=12)		II(n=14)		III(n=7)	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
E_h (mV)[1]	259	54	212	38	167	48	271	45	193	54	194	43	272	43	211	50	180	29
ΔE (mV)[2]	78	23	64	35	38	26	87	30	66	43	65	50	89	20	53	26	42	13
rH[3]	23.33	1.84	21.65	1.32	20.40	1.65	23.64	1.55	21.10	1.90	21.27	1.48	23.77	1.78	21.73	1.68	20.49	1.41
t (min)[4]	6.2	3.3	6.6	3.5	6.0	2.6	6.1	2.4	6.0	2.4	6.7	2.7	5.0	2.2	4.8	1.3	7.4	6.5

[1] Measurements performed 5 sec after inserting the electrodes into the sample (platinium and calomel electrodes)

[2] The difference between the values obtained at 5 sec and at 3 min after the start

[3] $rH = \frac{2 \cdot E_h}{0.1984 \cdot T} + 2$ pH, where T = 293 °C and E_h is in mV

[4] The average time needed for the collection of a 10 ml saliva sample

Table V. *A comparison between reported values of the oxidation-reduction potential in human saliva*

E_h (mV) $\bar{x}$	S.D.	Remarks	References
+ 261	288	Mean value of unstimulated saliva of five individuals	*Eisenbrandt* (1943), quoted by *Burnett & Scherp* (1968)
+ 301		As above (seven individuals) Range + 158 — + 542	
+ 309	4.7	Stimulated saliva (50 caries-resistant persons)	*Eggers-Lura* (1955), quoted by *Burnett & Scherp* (1968)
+ 237	9.9	Stimulated saliva (50 caries-active persons)	
+ 332	74	Stimulated saliva (46 persons) after neglection of oral hygiene for four days	*Scheinin & Mäkinen* (1971)
+ 401	40	Plaque suspensions (plaque collected in situ after four days neglection in oral hygiene)	
+ 272	43	Stimulated saliva (33 persons) after neglection of oral hygiene for 12—24 hours	The present paper, (sucrose group, subgroup I)

reducing values (Table IV). There were no measurable differences in the rate of the change between the sugar groups. It was obvious, however, that the salivary redox potential was affected by several factors of which the time of day when the collection was performed, was noticeable.

DISCUSSION

1. *Redox potential*

The literature available provides the following information on the oxidation-reduction potential of human saliva (Table V). A typical aerobic culture broth may have an E_h of + 300 mV. Cultures which produce hydrogen peroxide may have an oxidation-reduction potential as high as + 429 mV (*Burnett & Scherp*, 1968).

The connection between the salivary redox potential and oral health is still obscure (*c.f.* Table V). It can be assumed that the redox potential of an oral fluid sample would be higher (more oxidized) if there are low numbers of anaerobic lactobacilli or other anaerobic micro-organisms present in the oral cavity. In spite of positive redox potentials of oral fluid samples, there is obviously a negative E_h in the sulcus and interproximal area because of a great number of anaerobic bacteria in these areas.

It can also be assumed that there would be a positive correlation between the activity of oxidizing enzymes, e.g. lacto-peroxidase, and redox potential in human saliva. No such correlation could be found, however, between the sugar groups in this study.

2. *Peroxidases and the auxiliary ions*

The peroxidase activity of human whole mouth saliva is most likely largely derived from the two major salivary glands, (submaxillary and parotid glands) (*Nickerson, Kraus & Perry*, 1957; *Nickerson & Kraus*, 1955). The serous acinar cells of these glands seem to be a rich source of tissue specific peroxidase. It is generally maintained that bacterial enzymes do not strongly contribute to the peroxidase

activity of human saliva (*Nickerson, Kraus & Perry*, 1957). It is likely that other salivary glands are also involved in the peroxidase production, although their significance in this sence may not be noticeable. Peroxidase activity has been detected, for example, in sublingual glands (*Morrison et al.*, 1965). It is also known that a smaller portion of the activity demonstrable with guaiacol is localized in granules of neutrophilic and eosinophilic leucocytes (representing the myeloperoxidase fraction) (*Nickerson & Kraus*, 1955).

Pseudoperoxidase activity of hemoglobin, myoglobin, ferritin, ceruloplasmin, cytochrome c etc. may be present in human whole mouth saliva in low amounts. Because the glandular lactoperoxidase will catalyze the oxidation of guaiacol 10^6 times more rapidly than will hemoglobin and cytochrome c (*Hosoya & Morrison*, 1965) and because the concentration of these pigments and other metal-containing proteins is not high in human whole mouth saliva, the possible contribution of pseudoperoxidase activity to the phenomen described in the present paper could be excluded.

It has been claimed that the salivary lactoperoxidase of the supernatant fluid of human whole mouth saliva comprises at least two major active peroxidases (A and B) which have different protein characteristics. Both forms contribute to the inhibition of bacterial growth (*Iwamoto et al.*, 1968). Human parotid saliva peroxidase also displays heterogeneity (*Iwamoto et al.*, 1972). Isoelectric focusing of human whole saliva has yielded three peroxidases demonstrable with guaiacol (*Iwamoto et al.*, 1972).

The heterogeneity of the salivary peroxidases has also been observed in their ability to inhibit the growth of bacteria in the presence of thiocyanate and hydrogen peroxide.

Dogon and *Amdur* (1970) suggested that two thiocyanate-dependent antibacterial systems are present in human parotid saliva. The first consists of SCN^- ions and an unidentified salivary protein (*Dogon, Kerr & Amdur*, 1962; *Dogon & Amdur*, 1965). The SCN^- ions were claimed to react with an acceptor within the bacterial cytoplasm under the influence of a salivary enzyme. The factor was effective only on actively growing organisms(*Zeldow*, 1963). The second system consists, according to *Dogon & Amdur* (1970), of lactoperoxidase, SCN^- ions, and H_2O_2, as also described by *Klebanoff* and *Luebke* (1965). The present information suggests that the first-mentioned system may also represent a lactoperoxidase-containing factor. Lactoferrin, an iron-binding protein also present in milk, has been suggested to occur in saliva and other body secretions (*Masson, Heremans & Dive*, 1966). For the molecular weight of the salivary peroxidase a value of 78000 has been reported (*Morrison, Bayse & Danner*, 1970). A value of 73500 was obtained in the present paper for the corresponding enzyme. For myeloperoxidase the corresponding value is 149000 (*Morrison et al.*, 1970) (152000 in the present case for an enzyme with corresponding properties). For a whole mouth saliva antibacterial factor which most likely corresponded to a peroxidase, a value of 80000—100000 was obtained (*Iwamoto & Matsumura*, 1966). Thus, the molecular weights determined in the present study are quite well within the range of values earlier presented for salivary peroxidases.

Iwamoto & Matsumura (1966) have found only one single peroxidase peak demonstrable with guaiacol and CM-cellulose chromatography. This peroxidase

most likely corresponded to Peak II of the present study. The preparation used by the above authors was, however, treated with ammonium sulphate before chromatography, which may have removed from the material studied an enzyme corresponding to Peak I of the present paper. The present paper additionally provided the following important information: Peak II was practically totally missing in the salivary sediment material. This material only yielded a peroxidase corresponding to Peak I of the whole saliva supernatant fluid. Moreover, the activity of Peak I of the supernatant fluid fractionations was very low in the fructose and xylitol groups. The activity was also rather low in the sucrose group.

According to *Morris, Weast* and *Lineweaver* (1946), the use of guaiacol as the lactoperoxidase substrate effectively inhibits the catalase possibly simultaneously present. Furthermore, the material tested in the present paper did not display any measurable catalase activity. Consequently, the differences in the peroxidase activities between the sugar groups could not be attributed to the possible reduction of the concentration of H_2O_2 in the sucrose and fructose groups, as a result of a catalase reaction. Catalase activity has earlier been detected in human saliva, but the material investigated was not centrifuged (*Kraus, Perry & Nickerson*, 1958) or the assay method may have been coarse (*Deakins*, 1938, 1941). The present study showed practically no catalase activity in *centrifuged* whole mouth saliva (stored a few weeks at —20°C).

The concentration of ionized iodine and thiocyanate was practically the same in all three sugar groups. This indicates that the above mentioned differences between the lactoperoxidase activities were not due to different concentration of these auxiliary factors in the samples studied. Furthermore, after chromatography (desalting on Sephadex G-25 and CM-chromatography) the possible minor differences with regard to the factors mentioned would anyway have disappeared.

The inhibition of Peak II by 10^{-4} M CN^- ions further proved its close identity with lactoperoxidase. The cyanide effect differentiates peroxidases of different sources (*Nickerson & Kraus*, 1955). CN^- ions inhibited totally the salivary peroxidase (Peak II) of the present paper, but not the activity of Peak I of the fructose and sucrose material. Because Peak I of the xylitol group was also totally inhibited, this enzyme preparation may not have contained bacterial peroxidases to any noticeable extent.

Peak II (the salivary lactoperoxidase) was totally missing in the salivary sediment material which contains bacteria and leucocytes (Figs. 1d, e, f). Nor was this enzyme present in serum, plaque and gingival exudate material which would possible have contributed to the salivary peroxidase activity. This enzyme was clearly present in higher amounts only in fractionations of the supernatant fluid of whole mouth saliva.

Based on the above references, on the fractionation patterns obtained in this paper, as well as on the molecular weight determinations and chemical characteristics revealed, it seemed likely that Peak I of the present study represented a mixture of several peroxidases. This peak may have contained traces of bacterial peroxidases (liberated by sonication), myeloperoxidase and pseudoperoxidase activity. It also may have contained heterogenic forms of the salivary lactoperoxidase. It was, however, the nature and behavior of Peak II, rather than those of Peak I, which were considered more interesting. The increased

activity of peroxidase (Fig. 1) in the supernatant fluid of whole mouth saliva of the xylitol-consuming test persons was most likely due to the involvement of this particular peroxidase. All literature information available and the results of this paper suggest that the elevation of the activity was caused by the involvement of the salivary lactoperoxidase, the very enzyme secreted from the major salivary glands.

The possible biological significance of the phenomenon described in the present paper should be emphasized. It may be expected that the qualitative and quantitative enzyme spectrum of human saliva is subjected to continuous and considerable variation not only as a result of sex, season and time of day, but also as a result of the chemical nature of the carbohydrates and other nutritional ingredients consumed by man. Related thoughts have been presented already half a century ago for the salivary amylase (*Walker*, 1925, quoted by *Afonsky*, 1961). *Ferguson* (1975) has demonstrated a negative feedback system containing dietary proteins, gastrin and cyclic AMP to control the salivary α-amylase levels. The effect of dietary sugars on the production of salivary enzymes seems to be selective. In the case of peroxidases the proved action of them in the natural defence mechanism in inflammation may evoke positive anticipations with regards to the control of certain inflammatory processes in the human oral cavity. In this context it is important to emphasize the proved correlation between the caries inhibitory and lactoperoxidase-increasing effects of xylitol (*Scheinin, Mäkinen & Ylitalo*, 1974, 1975).

The somewhat lower concentration of ionized fluorine in the saliva of the xylitol-consuming persons when compared to other groups is most likely reflected in the clinical condition of the subjects. Lower caries incidence, lower lactate concentration in plaque, and an insignificant extent of demineralization in the xylitol group, seem to keep fluorine in tissues (e.g. enamel) where it should occur in higher amounts.

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